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An *in vitro* analysis of striatal microcircuitry

By

Rasha ElGhaba

A thesis submitted to the University of Sheffield in fulfilment of the requirements for
the degree of Doctor of Philosophy

July 2015



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An *in vitro* analysis of striatal microcircuitry

By

Rasha ElGhaba

A thesis submitted to the University of Sheffield in fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Sheffield

Faculty of Science

Department of Psychology

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Summary

Low threshold spiking interneurons (LTSIs) and cholinergic interneurons are subtypes of striatal neurons that play an important role in modulation of final striatal output. This thesis describes an in vitro electrophysiological investigation of the mutual control of LTSIs and cholinergic interneurons. Moreover, this thesis describes the effects of three classes of opiate receptors agonists on LTSIs and cholinergic interneurons were investigated. Experiments were carried out using transgenic NPY-GFP mice or transgenic ChR2-EYFP-SOM-IRES-Cre mice. Whole-cell, perforated-whole cell and cell-attached recordings were obtained from mice brain slices maintained in vitro.

Our data report the presence of both GABAergic and cholinergic tones controlling LTSIs. Endogenous acetylcholine modulates LTSIs both directly and indirectly. The main direct effect of endogenous acetylcholine is inhibition of LTSIs through muscarinic cholinergic receptors. On the other hand, the main indirect effect of endogenous acetylcholine is activation of LTSIs. Moreover, endogenous acetylcholine inhibits the GABAergic transmission on LTSIs.

Furthermore, blue light- activation of LTSIs depolarized the cholinergic interneurons. This depolarization was only blocked by NO synthase inhibitor (L-NAME; 100 μ M). These results indicate that the LTSIs exert a main stimulatory effect on the cholinergic interneurons mediated by nitric oxide through s-GMP independent mechanism.

Regarding the opioidergic control of LTSIs and cholinergic interneurons, our data report that δ receptor agonist (DPDPE; 1 μ M) and κ receptor agonist (U-50488 hydrochloride; 10 μ M) strongly inhibit LTSIs and cholinergic interneurons. Moreover, DPDPE inhibits GABAergic transmission on LTSIs but U-50488 does not affect the GABAergic transmission on LTSIs.

Furthermore, μ receptor agonist (DAMGO; 1 μ M) has dual effect on LTSIs. The dual effect persisted in the presence of TTX. When GABA_A, nicotinic and muscarinic receptors were blocked in presence of TTX, DAMGO always inhibited LTSIs. DAMGO also inhibited the GABAergic transmission on LTSIs.

These results cast the light on striatal microcircuitry related to LTSIs and cholinergic interneurons.

This Thesis is dedicated to those who believe that this planet can become a better place and invested their lives in proving it.

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List of Abbreviations

ACh	Acetylcholine
ACSF	Artificial cerebrospinal fluid
AHP	After-hyperpolarization
BAC	Bacterial artificial chromosome
BG	Basal ganglia
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
sGMP	Cyclic guanosine monophosphate
CR ⁺ neuron	Calretinin (CR)-expressing interneurons
CTOP	Selective μ receptor antagonist
DA	Dopamine
DAMGO	Selective μ receptor agonist
D-AP5	d(-)-2-amino-5-phosphonopentanoic acid
DPDPE	D-Pen ² ,5-enkephalin
DYN	Dynorphin
ENK	Enkephalin
EPSP	Excitatory postsynaptic potential
FSI	Fast spiking interneuron
GABA	γ -aminobutyric acid
GFP	Green fluorescent protein
GP(e/i)	Globus pallidus (external segment/internal segment)
hrGFP	humanized Renilla GFP
IPSP	Inhibitory postsynaptic potential
ISI	Inter-spike interval
LID	L-DOPA induced dyskinesia
LTSI	Low-threshold spiking interneuron
MSN	Medium spiny interneuron
NAc	Nucleus accumbens
NAcc	Nucleus accumbens core
nAChR	Nicotinic cholinergic receptor

NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulphonamide disodium salt hydrate
NGF	Neurogliaform interneurons
nNOS	neuronal nitric oxide synthase
NMDA	N-methyl-d-aspartic acid
NO	Nitric oxide
NPY	Neuropeptide Y
PANs	Physically active neurons
POMC	Proopiomelanocortin
PPR	Paired-pulse ratio
SDM25N	Selective δ receptor antagonist
sIPSP	spontaneous IPSP
sGC	soluble guanylyl cyclase
SN(c/r)	Substantia nigra (pars compacta/pars reticulata)
SOM	Somatostatin
SP	Substance P
sst	Somatostatin
STN	Subthalamic nucleus
TANs	Tonically active neurons
U50488H	Selective κ agonist
VP	Ventral pallidum
VTA	Ventral tegmental area

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Chapter One: General introduction

The basal ganglia comprise a group of subcortical interconnected nuclei situated at the base of the forebrain. In humans, many disorders are associated with malfunction of the basal ganglia such as Parkinson's disease, Tourette's syndrome, Huntington's disease, attention-deficit disorder, obsessive-compulsive disorder, schizophrenia, and addictions. Observation of these conditions highlighted the importance of studying the physiology of the basal ganglia (Redgrave, 2007; Heinz Steiner & Kuei Y Tseng, 2010).

Furthermore, the striatum is largest nucleus and main input structure of the basal ganglia. This thesis reports on an experimental investigation into the mutual interaction between two striatal interneurons. These interneurons are the low threshold spiking interneurons (LTSIs) and the cholinergic interneurons. Moreover, we investigated the effects of different subtypes of opiate peptides on the activity of LTSIs. The general introduction provides background information on the basal ganglia, with particular focus on the striatum and its neurons. It then describes what was known about the role of three transmitters (acetylcholine, NO and opioids) in the striatum. This information aims at giving a clear picture about the topics of this research.

1.1 The Basal Ganglia

In this section, I will first present a brief general description of the anatomy, physiology, and function of the basal ganglia. Then, the striatum and striatal interneurons will be discussed in more details.

1.1.1 Anatomy of the basal ganglia

Anatomically, the basal ganglia are consisting of four main nuclei: the striatum, globus pallidus, substantia nigra, and subthalamic nucleus (Figure 1.1). The striatum is divided into: the ventral striatum and the dorsal striatum (neostriatum). The globus pallidus (or pallidum) is divided into three functional parts: pars externa (GPe), pars interna (GPi), and the ventral pallidum (VP) (Gerfen & Wilson, 1996). Finally, the substantia nigra consists of pars compacta (SNc) and pars reticulata (SNr) (Garrett E Alexander, DeLong, & Crutcher, 1992; Kandel, Schwartz, & Jessell, 2000).

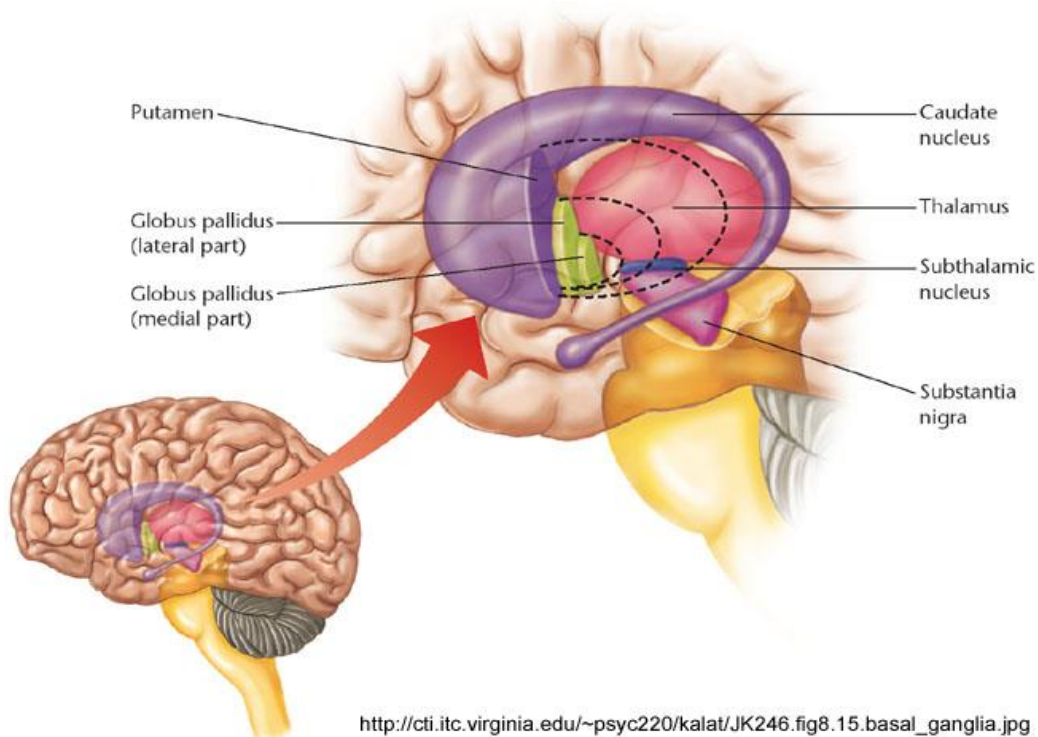


Figure 1.1: Anatomical organization of the basal ganglia.

The basal ganglia are subcortical nuclei consisting of: (1) the striatum which is divided into ventral and dorsal striatum. In primates, the internal capsule divides the dorsal striatum into caudate and putamen nuclei, (2) globus pallidus, (3) substantia nigra, and (4) subthalamic nucleus.

(Adapted from: http://cti.itc.virginia.edu/~psyc220/kalat/JK246.fig8.15.basal_ganglia.jpg)

1.1.2 Physiology of the basal ganglia (Figure 1.2)

The basal ganglia have two input nuclei and two output nuclei. The striatum represents the first and main input nucleus of the basal ganglia. The striatum receives glutamatergic inputs from most regions of the cerebral cortex, limbic system, and brainstem (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004). Moreover, the striatum receives dopaminergic inputs from SNc and serotonergic inputs from the raphe nuclei in the midbrain (Yelnik, 2002). The subthalamic nucleus (STN) is the second input nucleus. It receives major inputs from cortical and sub-cortical areas (thalamus and brainstem). Additionally, STN receives modulatory dopaminergic, serotonergic and cholinergic inputs (Mena-Segovia, Bolam, & Magill, 2004; Mouroux & Feger, 1993).

On the other hand, the internal globus pallidus (GPi) represents the first output nucleus in the basal ganglia. The GPi receives excitatory glutamatergic input from the STN and inhibitory GABAergic afferents from the striatum and GPe. This GPi sends powerful inhibitory impulses to the brainstem and the thalamus (Mena-Segovia et al., 2004). The substantia nigra pars reticulata (SNr) is second output structure of the basal ganglia. The SNr receives inputs from the other nuclei of the basal ganglia then it sends strong inhibitory outputs to the thalamus and brainstem (G. Chevalier & J. Deniau, 1990; G. Chevalier & J. M. Deniau, 1990).

Signals are processed in the basal ganglia through two main pathways: the direct pathway and the indirect pathway. Activation of these two pathways has opposite effects on the output target structures in the thalamus (Knierim, 1997).

The direct pathway begins in the striatum with neurons that send inhibitory output to the GPi. The GPi neurons also send inhibitory signals to the thalamic neurons. When the direct pathway striatal neurons fire, they inhibit the GPi neurons leading to release of the inhibition of the thalamic neurons (i.e. disinhibition of the thalamic neurons), allowing the thalamic neurons to activate the cortex. On the other hand, the indirect pathway begins with another group of striatal neurons that send inhibitory outputs to the external segment of the globus pallidus (GPe). When the GPe neurons fire they inhibit the subthalamic nucleus. The subthalamic nucleus sends excitatory output to the GPi. Thus, when the indirect pathway neurons are active, they will inhibit the GPe neurons. Thus, the subthalamic nucleus will be active leading to activation of the GPi, thereby inhibiting the motor cortex (Knierim, 1997).

Thus the direct and indirect pathways act differentially to modulate the inhibitory outflow from the basal ganglia output nuclei. The activation of the direct pathway facilitates the motor movement through disinhibition of the inhibitory effect of GPi/SNr. On the other hand, activation of the indirect pathway will act to suppress any unwanted sequence of movement (Mink & Thach, 1993). A balance between the activity of the direct and indirect pathways is important for the basal ganglia to function normally.

It also worth mentioning that the basal ganglia projections to the thalamus project back to the striatum and STN (Kimura, Minamimoto, Matsumoto, & Hori, 2004; Smith, Beyan, Shink, & Bolam, 1998). Similarly, basal ganglia projections to the brainstem send indirect inputs to the striatum through the midline and intralaminar nuclei of the thalamus (Fadila, 2004).

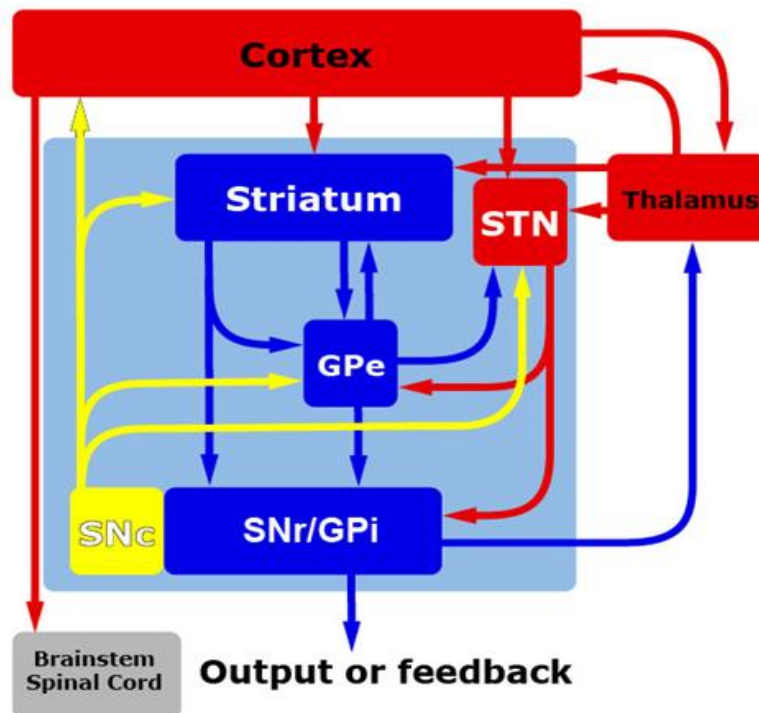


Figure 1.2: Basal ganglia input and output structures

The basal ganglia input nuclei; the striatum and the subthalamic nucleus (STN) receive glutamatergic inputs from the cortex and the thalamus. They also receive modulatory inputs from the SNc and raphe nuclei. The striatum projects to the SNr and GPi output nuclei. The output nuclei project back to the thalamus and the cortex. The blue box include the nuclei of the basal ganglia. Red arrows; glutamatergic projections. Blue arrows; GABAergic projections. Yellow arrows; dopaminergic projections. Adapted from (J. Tepper, Abercrombie, & Bolam, 2007)

1.1.3 Functions of the basal ganglia

The basal ganglia are implicated in many important functions including; motor, sensory, limbic (reward), and cognitive (associative) functions (Redgrave, 2007; H. Steiner & K. Y. Tseng, 2010). It was originally proposed that basal ganglia process cortical information for these different functions through segregated parallel circuits including motor, sensory, limbic (reward), and cognitive (associative) ones (G. E. Alexander & Crutcher, 1990). Now, it is believed that there is integration between these parallel functional circuits inside the basal ganglia (Figure 1.3) (Clarke, Bevan, Cozzari, Hartman, & Bolam, 1997; Draganski et al., 2008; Redgrave, 2007).

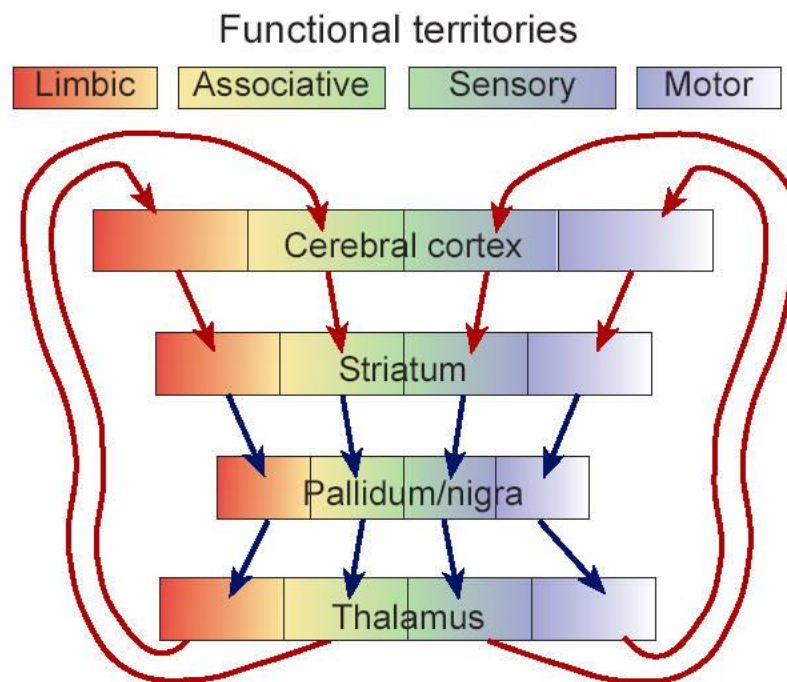


Figure 1.3: A proposed model of the basal ganglia processing of information.

The connections between the functional territories in cerebral cortex, the basal ganglia and the thalamus occur mainly through parallel segregated loops. Adapted from (Redgrave, 2007).

1.1.3.1 Motor function of the basal ganglia

The pioneering work of DeLong (1971) showed that activity in the basal ganglia is correlated with movement (DeLong, 1971). Since then research was carried out to identify the nature of motor signals coded by the basal ganglia.

It is believed that the basal ganglia gate the execution of automatic motor programs mainly through suppression of the inappropriate motor acts during the execution of specific movement. This motor function of the basal ganglia can be easily understood through the action selection property of the basal ganglia (Redgrave, 2007).

1.1.3.2 Cognitive function of the basal ganglia

Beside the motor function, the basal ganglia play an important role in the selection between different cognitive and emotional programs stored in the cerebral cortex. Moreover, the basal ganglia are involved in reinforcement learning. Furthermore, in humans, the basal ganglia are necessarily involved in some tasks of implicit memory (Wolfram Schultz, 2006).

1.1.3.3 Reward function of the basal ganglia

Rewards are any stimuli or events characterized by: (1) generation of approach or consummatory behaviour, (2) an increase in the probability of certain behaviour to occur (learning or positive reinforcement), and (3) induction of subjective feelings of pleasure and hedonia (W Schultz, 2007). Rewards can be classified into primary and secondary rewards. Primary rewards include stimuli or events necessary for the survival of species, such as feeding, drinking, and reproduction, while, secondary rewards usually drive their value from primary rewards such as money (M. R. Delgado, 2007).

The basal ganglia play a fundamental role in reward processing in the brain. The basal ganglia act initially to integrate the sensory information with reward value, and memory. Then, the basal ganglia incorporate this information with cognition which helps in developing a motor plan. Finally, the basal ganglia provide the motor control to execute target behaviour (M. R. Delgado, 2007).

Several nuclei of the basal ganglia share in reward processing such as the striatum especially ventral striatum, substantia nigra and ventral pallidum. Reward processing in the basal ganglia is fundamental for learning about selection of appropriate action and habit formation (M. R. Delgado, 2007; W. Schultz, 2000).

On the other hand, drugs of abuse act to hijack the reward system in the brain by mimicking natural reward stimuli (Koob & Le Moal, 2008). These drugs stimulate neuronal pathways responsible for natural reward then progressively the drugs act to alter the function of these pathways (Everitt & Robbins, 2005; Hyman, Malenka, & Nestler, 2006; Kelley & Berridge, 2002).

1.2 The striatum

As mentioned before, the striatum is main input nucleus of the basal ganglia. The striatum is involved in many aspects of learning, such as habit formation (Jog, Kubota, Connolly, Hillegaart, & Graybiel, 1999), skill learning (Poldrack, Prabhakaran, Seger, & Gabrieli, 1999), and reward-related learning (O'Doherty, 2004). Therefore, it was postulated that striatum integrate cognition, motor control, and motivation information. In the following section, an overview on the striatum will be given. This overview will include the anatomy, the compartments of the striatum and the role of the striatum in the reward processing.

1.2.1 Organization of the striatum

As stated earlier, the striatum has two main subdivisions: the dorsal and ventral striatum (Figure 4). In primates, the dorsal striatum consists of the caudate and the putamen which are separated by the internal capsule. In rodents there is no clear separation between these two nuclei (H. Steiner & K. Y. Tseng, 2010). Although the presence of these anatomical differences but functional organization is nearly identical between rodents and primates.

The dorsal striatum can be further divided into the dorsomedial striatum which receives innervations mainly from the cortical association areas (Goldman & Nauta, 1977; A. McGeorge & R. Faull, 1989), and, the dorsolateral striatum which receives innervations from sensorimotor cortex (Künzle, 1975; A. J. McGeorge & R. L. M. Faull, 1989).

On the other hand, the ventral striatum consists of the nucleus accumbens, which is composed of core and shell regions. The core region resembles the dorsal striatum, while the shell region resembles the amygdale (Zahm, 2000). Recently, studies proved that ventral striatum does not only include the nucleus accumbens but it extend more dorsally and caudally into the ventral parts of caudate and putamen nuclei (M. R. Delgado, 2007; Gottfried & Haber, 2011; Packard & Knowlton, 2002). The ventral striatum is innervated with glutamatergic afferents from frontal cortex and limbic regions (J. S. Brog, A. Salyapongse, A. Y. Deutch, & D. S. Zahm, 1993), and dopaminergic afferents from the ventral tegmental area (Fields, Hjelmstad, Margolis, & Nicola, 2007).

1. 2.2 Striatal patch/matrix compartments

Although the striatum appears uniform under the light microscope ; however, developmental studies proved that striatum is composed of two compartments ; the patch (or striosome) and matrix compartments (Figure 1.4) (A. M. Graybiel & Ragsdale, 1978). This patch/matrix organization was confirmed by staining with neurochemical markers such as acetylcholinesterase and immunohistochemical studies such as opiate receptor binding.

Patches represent 10% of total volume of striatum. The striatal patches are characterized by rich μ -opioid receptor binding, substance P staining, and poor staining for cholinergic markers. On the other hand, the matrix is characterized by dense acetylcholinesterase and cholineacetyltransferase staining (A. Graybiel, Baughman, & Eckenstein, 1986), and immunoreactivity for calbindin and somatostatin (Charles R Gerfen, 1992).

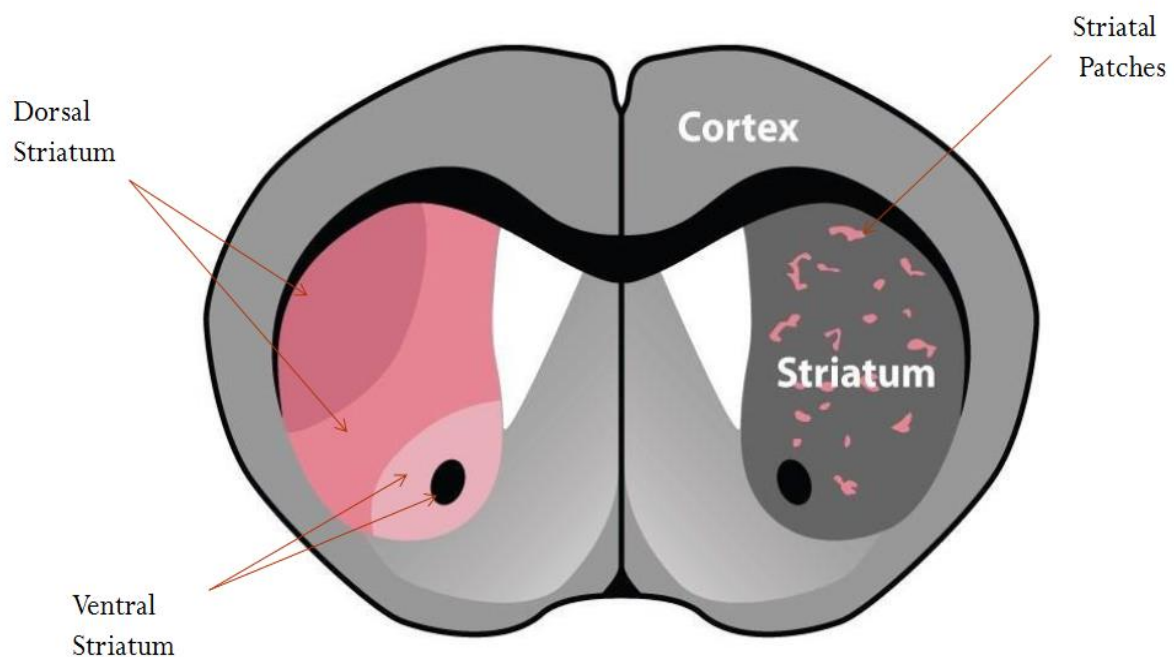


Figure 1.4: Organization of the striatum.

Coronal section of rat forebrain. Left hemisphere: anatomical divisions of the striatum include: dorsal; dorsolateral (dark pink), dorsomedial (moderate pink), and ventral (light pink) striatum. The core of the nucleus accumbens is shown as a black circle. Right hemisphere shows patch/matrix compartments of the striatum: striatal patches (pink) where dense μ -opioid receptor binding exists. Adapted from (Kreitzer, 2009).

1. 2.3 Role of striatum in reward processing

It was proved through studies across species with different methodologies that the striatum plays an important role in reward function in the brain. Studies on rodents have shown that the ventral striatum, especially the nucleus accumbens, is involved in reward processing. On the other hand, the dorsal striatum plays an essential role in learning and updating of reward actions. Lesions in the rat ventral striatum are associated with deficits in approach behavior, while, lesions in the rat dorsal striatum are associated with failure of reward consumption (Apicella, Scarnati, Ljungberg, & Schultz, 1992; O'Doherty, 2004; Tricomi, Delgado, & Fiez, 2004).

Moreover, studies in primates suggested that striatal neurons respond to the anticipation and delivery of rewards (Paul Apicella et al., 1992; Kawagoe, Takikawa, & Hikosaka, 1998). It was found that the striatal neurons fired more vigorously with preferred rewards (Hassani, Cromwell, & Schultz, 2001). Moreover, the activity of striatal neurons is modulated with different magnitudes of reward (Cromwell & Schultz, 2003). Furthermore, self-administration of cocaine in rats was associated with increased DA release in both dorsal and ventral striatum (Di Chiara & Imperato, 1988).

Furthermore, electrophysiological recordings showed that the dopaminergic neurons fire after the delivery of unexpected rewards. After learning, these neurons fire to the earliest predictor of rewards (e.g., a light). Moreover, when an unpredicted reward fails to occur, dopaminergic neurons decrease in firing. These results suggest that dopaminergic neurons play a role in coding for prediction errors during affective learning (Wolfram Schultz, Dayan, & Montague, 1997). In humans, prediction errors were associated with increased activity in the dorsal and ventral striatum (McClure, Berns, & Montague, 2003; Pagnoni, Zink, Montague, & Berns, 2002).

From another prospective; neuroimaging studies in human reported an increase DA release in the dorsal and the ventral striatum with food rewards. In one study, food was able to increase the DA in the dorsal striatum of hungry participants (Volkow et al., 2002). By using functional magnetic resonance imaging (fMRI) in studying the human brain's reward circuit and addiction, Breiter and colleagues showed that injections of cocaine in cocaine addicts led to activation of the ventral striatum correlated to the feelings of craving, while the dorsal

striatum was activated after drug injection in correlation to the feelings of the rush (Breiter et al., 1997).

Now, it is suggested that the striatum plays an essential role not only in reward processing, but also in aversive processing (Breiter et al., 1997; Jensen et al., 2003; Seymour et al., 2004).

Striatum consists of many types of neurons. These neurons process information inside the striatum. In the following section, an introduction on different striatal neurons will be presented. The physiology of each type of the striatal neurons will be discussed. This aims at explaining the background related to this research project.

1.3 Striatal neurons

Striatal neurons can be divided into two main categories: the projection neurons and the striatal interneurons.

1.3.1 Projection neurons (medium spiny neurons ;MSNs)

MSNs represent 95% of striatal neurons. MSNs are GABAergic neurons that receive glutamatergic innervations from cortex and thalamus (Kemp & Powell, 1971). MSNs also receive dopaminergic innervations from the midbrain (Smith, Bennett, Bolam, Parent, & Sadikot, 1994).

Electrophysiological properties of MSNs

Electrophysiological properties of the MSNs include: relatively hyperpolarized resting membrane potential and a low input resistance (Kita, Kita, & Kitai, 1984). MSNs are characterized by many potassium conductances that lead to their unique pattern of firing (Nisenbaum & Wilson, 1995) (Figure 1.5).

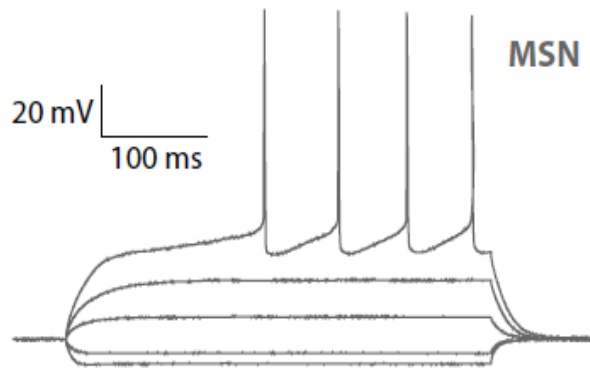


Figure 1.5: Electrophysiological properties of striatal MSNs

The MSNs responses to depolarizing and hyperpolarizing currents show that the MSNs exhibit hyperpolarized resting membrane potential, low input resistance, and slow depolarization. Adapted from (Kreitzer, 2009).

Many studies noted that striatal MSNs exhibit fluctuations in the membrane potential between hyperpolarized state -90 to -70 mV and depolarized state -60 to -40 mV (C. J. Wilson & Groves, 1981). This condition is known as Up and Down states which arise as a result of the intrinsic MSNs membrane properties (Mahon et al., 2006).

Divisions of striatal MSNs

The MSNs are divided into two main populations that differ in their connections and neurochemistry (Figure 1.6):

- a. **Striatonigral (direct-pathway) neurons** which directly project to the basal ganglia output nuclei in the internal pallidum (GPi) or the substantia nigra pars reticulata (SNr). Besides GABA, the direct pathway MSNs contains substance P (SP) and dynorphin (DYN). Moreover, The direct-pathway MSNs express dopamine D1 and muscarinic M4 receptors (Ince, Ciliax, & Levey, 1997; Longsta, 2000). Activation of the direct pathway neurons inhibits the GABAergic output of GPi and SNr to the thalamus. This increases the thalamocortical activity which facilitates movement (Kandel et al., 2000; Longsta, 2000).

- b. Striatopallidal (indirect-pathway) neurons** which project to the external pallidum (GPe) then to the STN. STN activates the inhibitory GABAergic neurons in the GPi and SNr. The indirect pathway neurons contain GABA and enkephalin (ENK) as neurotransmitters. These MSNs express D2 dopamine and adenosine A2A receptors(Charles R Gerfen, 1992). Activation of this pathway results in decreased firing of thalamic neurons which inhibits movement (Kandel et al., 2000; Longstaff, 2000).

However, it was discovered that the neurons of the direct pathway send collaterals to the GPe. Thus the direct pathway neurons also contribute to the indirect pathway system (Parent et al., 2000) (Figure 1.6).

Recently, optogenetics were used for direct activation of MSNs of direct and indirect pathway in vivo. It was found that bilateral activation of MSNs of indirect pathway increased freezing and bradykinesia. On the other hand, excitation of MSNs of direct pathway decreased freezing and enhanced locomotor initiation (Kravitz et al., 2010).

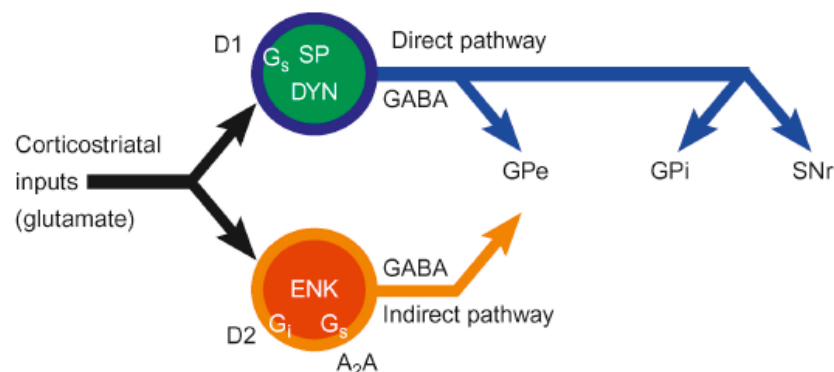


Figure 1.6: The striatonigral (direct) and striatopallidal (indirect) pathways.

Direct and indirect pathways neurons receive glutamatergic inputs from cortex and the thalamus. Striatonigral neurons express D1 receptor, substance P (SP) and dynorphin (DYN). Then these neurons project to the GPi, SNr and GPe. On the other hand, striatopallidal neurons express D2 receptor, A2A receptor, and enkephalin (ENK). These neurons project to the GPe. Adapted from (H. Steiner & K. Y. Tseng, 2010).

Regarding patch/matrix compartments, it was found that MSNs in the matrix receive innervations from cortex and thalamus (Sadikot, Parent, Smith, & Bolam, 1992). The matrix MSNs send outputs to the SNr and the GP (C. R. Gerfen, 1992). While, patches MSNs receive inputs mainly from limbic and frontal cortex (Kincaid & Wilson, 1996).

Neuromodulation of projection neurons

Striatal MSNs are sensitive to numerous neuromodulators such as dopamine and acetylcholine. Dopamine activation of D1 receptors reduces MSNs excitability. However, if the MSNs are in the Up state, D1 receptors activation facilitates their firing (Lacey, Mercuri, & North, 1990; Uchimura & North, 1990). On the other hand, D2 receptor activation decreases MSN firing (Hernández-López et al., 2000).

Furthermore, Acetylcholine activates M1 receptors expressed by striatal MSNs (Yan, Flores-Hernandez, & Surmeier, 2001). Stimulation of M1 receptors shifts striatonigral MSNs membrane potential to more hyperpolarized state (Akins, Surmeier, & Kitai, 1990). On the contrary, M1 receptor activation in the striatopallidal MSNs increases MSNs excitability and spiking (Shen, Hamilton, Nathanson, & Surmeier, 2005).

1.3. 2 Striatal interneurons.

Striatal interneurons represent a minority (4%) of striatal neurons. These interneurons receive glutamatergic afferents from cortex and thalamus. The output of these interneurons is mainly directed to MSNs and other interneurons. Thus these interneurons form microcircuits that modulate final striatal output (J. M. Tepper & Bolam, 2004). These interneurons are now in the focus of many electrophysiological studies that are trying to explore their physiological characteristics as well as the action of different neurotransmitters on their function.

1.3.2.1 Nitroergic low-threshold spiking interneurons (LTSIs)

LTSIs represent a subtype of striatal interneurons. LTSIs are considered the second largest cell in the striatum (Kawaguchi, 1993). The LTSIs express many neurotransmitters (i.e. GABA, somatostatin, NPY, and NO) in the striatum (Kawaguchi, Wilson, Augood, & Emson, 1995). These transmitters play an important role in controlling of striatal excitability.

Striatal LTSIs receive glutamatergic afferents from the cortex (Kawaguchi, 1993) and dopaminergic afferents from substantia nigra (Yoshiyuki Kubota et al., 1988). Also, LTSIs receive inputs from other striatal interneurons as FS and cholinergic interneurons (Bevan, Booth, Eaton, & Bolam, 1998).

Electrophysiological properties of LTSIs

LTSIs are one of tonically active neurons (TANs) which are characterized by being spontaneously active. These LTSIs are characterized by the presence of a low threshold Ca^{2+} spikes, hence their name (LTS) (D. Centonze et al., 2002). LTSIs are also characterized by a resting membrane potential of -56.4 ± 15.7 mV, long duration action potential (1.0 ± 0.41 ms at half amplitude), and a very high input resistance (638 ± 245 M Ω) (D. Centonze et al., 2002) (Figure 1.7). Furthermore, LTSIs exhibit different firing patterns such as regular, irregular or burst firing with ability of spontaneous transition between them (Beatty, Sullivan, Morikawa, & Wilson, 2012).

Previously, studies on striatal LTSIs were limited due to their small numbers and their undistinguishable morphology under microscopy (Kawaguchi, 1993; Y. Kubota & Kawaguchi, 2000). This problem was solved through developing of transgenic mice in which a humanized Renilla GFP (hrGFP) is expressed under the control of NPY promoter. This allowed easy identification of the LTSIs under fluorescent microscope (J. G. Partridge et al., 2009).

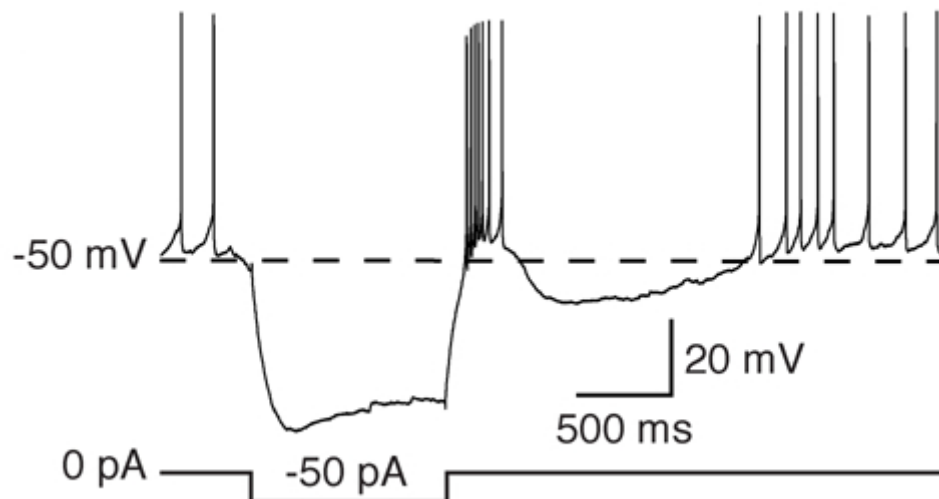


Figure 1.7: Electrophysiological properties of LTSIs.

LTSIs are spontaneously active neurons which are characterized by presence of a low threshold Ca^{2+} spikes, high input resistance, and long duration action potential. Adapted from (J. A. Beatty, M. A. Sullivan, H. Morikawa, & C. J. Wilson, 2012).

Neuromodulation of LTSIs

Neuromodulation of LTS interneurons by other neurotransmitters has not been well characterized. However, it is known that LTSIs express D5, M1 and M2 receptors (Ariano & Kenny, 1989; Rivera et al., 2002). It was found that D1-receptor agonists are able to depolarize LTSIs (D. Centonze et al., 2002).

Role of LTSIs in modulation striatal function

The LTSIs act primarily through modulation of striatal microcircuits. An earlier study reported that LTSIs make limited number of synapses with striatal MSNs cells (J. M. Tepper & Bolam, 2004). Moreover, using paired recording from LTSIs and MSNs, Koos and colleagues (1999) found that LTSIs exert an inhibitory effect on MSNs. This inhibitory effect was mediated by GABA (Koós & Tepper, 1999).

Somatostatin, secreted by the LTSIs, modulates the excitability of striatal projection neurons. It was reported that somatostatin modifies the firing pattern of MSNs through modulation of

MSNs Ca^{+2} activated K^{+} currents (Galarraga et al., 2007). Somatostatin also regulates the effectiveness of lateral inhibitory synaptic connections among MSNs. While, NPY share in the regulation of dopamine release in the striatum (Lopez-Huerta, Tecuapetla, Guzman, Bargas, & Galarraga, 2008).

LTSIs are considered to be the only known source for NO in the striatum. NO is an important neuromodulator for many neurotransmitters. It was shown that NO mediate different forms of synaptic plasticity, including short and long-term changes in the excitatory and inhibitory synaptic transmission (Susswein, Katzoff, Miller, & Hurwitz, 2004). More details about the role of NO in the striatum will be discussed later in this chapter.

1. 3.2.2 Cholinergic neurons:

Cholinergic interneurons represent 1-3% of striatal neurons. Cholinergic interneurons are characterized by a large cell body of 20–50 μm diameter with long aspiny dendrites (Kawaguchi, 1992) and large dense axonal collaterals with large terminal fields (J. Bolam, 1984). These criteria account for the high expression of ACh markers in the striatum (Butcher & Woolf, 1984). Moreover, The dense axonal arbors may play an important role through volume transmission (Koós & Tepper, 2002). It was reported that the dendrites of cholinergic neurons are widely distributed in the patch and matrix compartments in the striatum, whereas the dense axonal terminals are restricted to the matrix where they mainly target the MSNs (Kawaguchi et al., 1995).

Electrophysiological properties of cholinergic interneurons

Electrophysiologically, cholinergic interneurons are characterized by a resting potential of -62 ± 2.7 mV and high input resistance (Kawaguchi, 1993). There is a period of after hyperpolarization following each spike (Kawaguchi, 1992) (Figure 1.8).

Cholinergic interneurons are one of the tonically active neurons (TANs) in the striatum that fire at the rate of 2-10Hz (Bennett, Callaway, & Wilson, 2000). They act as autonomous pacemakers that fire spontaneously both in vivo and in vitro. Blocking of synaptic transmission does not stop the cholinergic spontaneous firing (Bennett et al., 2000). The firing rate of cholinergic interneurons widely differs ; however , it can be classified into three

main categories: an irregular single spiking, a rhythmic single spiking, and a rhythmic bursting pattern (F. M. Zhou, Wilson, & Dani, 2002). Bennett and his colleagues reported the ionic mechanism of the rhythmic single spiking pattern. It was found that the spontaneous firing occur as a result of a depolarization by a subthreshold Na^+ current followed by long after-hyperpolarization (AHP) due to activation of Ca^{2+} dependant K^+ current (Bennett et al., 2000). There are two factors that regulate the basal level of cholinergic signalling: the negative feedback via inhibitory muscarinic autoreceptors and the high levels of acetylcholinesterase, which restrict the cholinergic signalling both temporally and spatially within the striatum. However, the pacemaking activity of cholinergic neurons provides enough ACh levels to tonically activate muscarinic and nicotinic receptors in the striatum (Benarroch, 2012).

Furthermore, it was shown that cholinergic interneurons usually fire in burst-pause pattern during motor learning and reward-related behaviours (Paul Apicella, 2007).

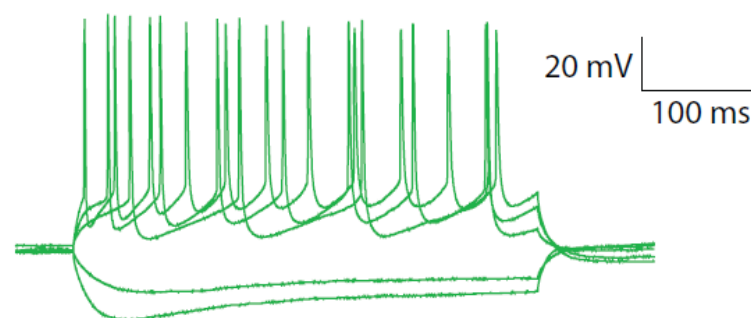


Figure 1.8: Electrophysiological properties of cholinergic interneurons.

Cholinergic interneurons responses to different depolarizing and hyperpolarizing currents show that these interneurons are characterized by spontaneous activity and broad spike with long spike after hyperpolarization. Adapted from (Kreitzer, 2009).

Neuromodulation of cholinergic interneurons (table1)

Anatomical studies proved that cholinergic interneurons receive more inputs from the thalamus compared to the cortex (S. Lapper & Bolam, 1992). Thus, the thalamostriatal pathway plays an important role in modulation of cholinergic interneurons activity.

Cholinergic interneurons receive inhibitory inputs from striatal MSNs (J. P. Bolam et al., 1986). Stimulation of muscarinic M2 and M4 receptors decrease the excitability of cholinergic interneurons (P. Calabresi et al., 1998). Moreover, M2 receptors act presynaptically to regulate ACh release (Hersch, Gutekunst, Rees, Heilman, & Levey, 1994).

Dopaminergic neuromodulation of cholinergic interneurons occur through activation D2 and D5 receptors. Activation of D5 receptor increase the excitability of cholinergic interneurons by increasing the cAMP (T. Aosaki, Kiuchi, & Kawaguchi, 1998). On the other hand, activation of D2 receptors decrease cholinergic interneurons excitability through inhibition of voltage-sensitive Na⁺ channels (Maurice et al., 2004). Finally, serotonin act on 5-HT_{2C}, 5-HT₆ & 5HT₇ to stimulate cholinergic interneurons (C. Blomeley & Bracci, 2005).

Table 1.1: The neuromodulation of cholinergic interneurons

Source	Neurotransmitter	Receptor	Effect
Cortex	Glutamate	AMPA & NMDA	Fast activation
Thalamus	Glutamate	AMPA & NMDA	Fast activation
FS interneurons	GABA	GABA _A	Fast inhibition
MSN	GABA Substance P	GABA _A Neurokinin-1	Fast inhibition Excitatory
Cholinergic interneurons	Acetylcholine	M2/M4 autoreceptors	Decrease of ACh release
Dopaminergic afferent	Dopamine	D2 D5	Inhibition of tonic firing Inhibition of ACh release. Depolarization and increase of ACh release.
Dorsal raphe	Serotonin	5-HT _{2C} , 5-HT ₆ & 5HT ₇	Excitatory

TAN pause response

In the in vivo studies, extracellular unit recording discriminate between two types of neurons in the striatum; the phasically active neurons (PANs) and the tonically active neurons (TANs) (Aosaki, Kimura, & Graybiel, 1995). The PANs are usually silent neurons; however their firing is only related to behavioural stimulation. The MSNs and most of GABAergic interneurons represent this category inside the striatum. On the other hand, TANs are characterized by autonomous activity. Moreover, antidromic stimulation of the globus pallidus does not affect TANs which indicate that these neurons are interneurons. Cholinergic interneurons and LTSIs belong to this category (Aosaki et al., 1995; Beatty et al., 2012).

For many years, in vivo studies focused on cholinergic interneurons as the main TANs inside the striatum. In this section we will review the TAN response in vivo taking in consideration that TANS include both the cholinergic interneurons and LTSIs.

It was found that the firing of TANs is not related to body movement per se; however, TANs seem to respond to sensory stimulation associated with reward (Kimura, Rajkowski, & Evarts, 1984). During sensorimotor acquisition, TANs develop a conditioned response (TAN pause response) that consists of an initial activation followed by a pause then a rebound activation (Figure 1.9) (Toshihiko Aosaki et al., 1994). Histological studies suggest that TANs that respond to any conditional stimulus are widely distributed in the striatum (A. M. Graybiel, Aosaki, Flaherty, & Kimura, 1994). Apicella reported that TANs response reflect the detection of the stimulus, the motor control and recognition of a specific behavioural value (Paul Apicella, 2007). It is now clear that TANs are key players in the selection of appropriate response to any environmental stimulus. Moreover, the TAN pause response is associated with an effect on dopamine release in the striatum. During the initial activation phase of cholinergic interneurons the dopamine levels drop in the striatum. On the other hand, during pause phase the dopamine levels increase (Toshihiko Aosaki, Miura, Suzuki, Nishimura, & Masuda, 2010).

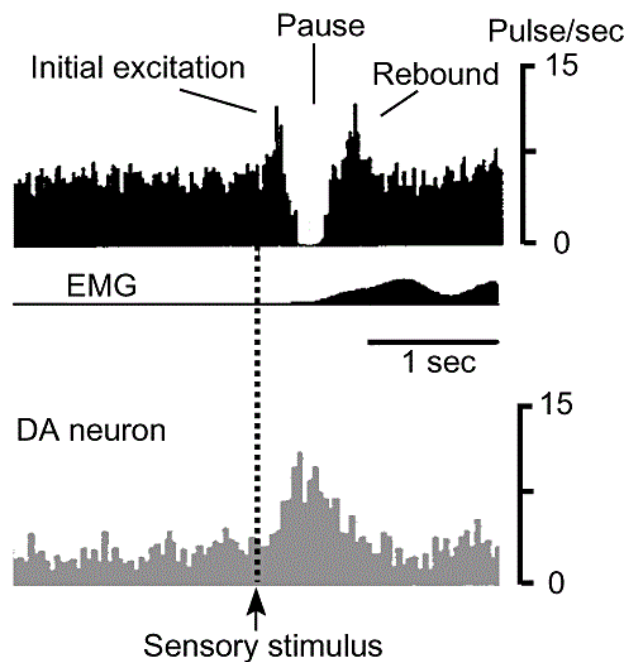


Figure 1.9: TAN pause response to salient stimuli.

Schematic drawings of TAN pause response. Salient stimulus elicits an initial excitation then a pause followed by rebound excitation. This response is associated with subsequent burst discharge of dopaminergic neurons in the SN. Adapted from (Toshihiko Aosaki et al., 2010).

Cellular mechanisms underlying TAN pause response

A salient stimulus usually causes an initial firing then transient cessation of tonic activity of TAN followed by rebound activation.

A. Initial excitation phase

In vivo studies reported that postsynaptic potentials associated with TAN pause response consist of an initial excitatory postsynaptic potential (EPSP) followed by GABAergic inhibitory component (IPSP). Application of glutamate blocker CNQX stopped not only the initial excitatory EPSP but also the subsequent GABAergic IPSP. This prove that the GABA component occur through a disynaptic mechanism (Toshihiko Aosaki et al., 2010).

B. Pause phase

In vivo recordings from the striatum showed the subthreshold and suprathreshold depolarizations of TANs led to subsequent afterhyperpolarization (AHP) (Reynolds, Hyland, & Wickens, 2004). The duration and amplitude of the AHP are directly proportional to the depolarization level. This process is thought to happen through GABA and dopamine D2 activation (Toshihiko Aosaki et al., 2010).

C. Rebound excitation phase

The pause of TANs firing is followed by a resumption of spontaneous firing that occur through activation of the Ih and the Na⁺ currents (Toshihiko Aosaki et al., 2010).

Physiological significance of TAN pause response

TAN pause response act as a time window for synaptic plasticity in the striatum (Miura, Saino-Saito, Masuda, Kobayashi, & Aosaki, 2007). Studies showed that the pause response is closely related to any conditional stimulus that predicts reward (Waelti, Dickinson, & Schultz, 2001). Beside the unpredicted conditional stimuli, TAN also responds to unexpected noxious stimuli (Ravel, Legallet, & Apicella, 1999). In this case, the pause response to noxious stimulus differs from the response to unpredicted reward (Zhou et al., 2002). Both types of TAN pause response indicate that TANs mainly respond to salient stimuli either it is rewarding or noxious. The response of TAN showed habituation if the stimulus was presented separately from process of learning (Sardo, Ravel, Legallet, & Apicella, 2000). Moreover, TAN pause response depend on the predictability so TAN respond to unpredicted stimulus. Once the stimulus become predicted , there is minimal or no response of TAN (Zhou et al., 2002).

1. 3.2.3 NPY- Neurogliaform interneurons:

Recently it was proved that LTS interneurons are not the only cells expressing NPY in the striatum. The NPY–neurogliaform (NPY–NGF) interneurons represent another subtype of NPY - interneurons which resemble electrophysiologically and morphologically the NPY GABAergic interneurons in cortex and hippocampus (O. Ibáñez-Sandoval et al., 2011).

NGF interneurons are characterized by their bright appearance with fluorescence imaging. In vitro electrophysiological recordings show that NPY-NGF interneurons are characterized by lower input resistance than LTSIs. The NGF resting membrane potential ranges between -95 to -79 mV, which is more hyperpolarized than that of LTS interneurons. NGF interneurons are not spontaneously active. They are also characterized by a long action potential. Finally, when a depolarizing current is injected the NGF fire regularly (O. Ibáñez-Sandoval et al., 2011) (Figure 1.10).

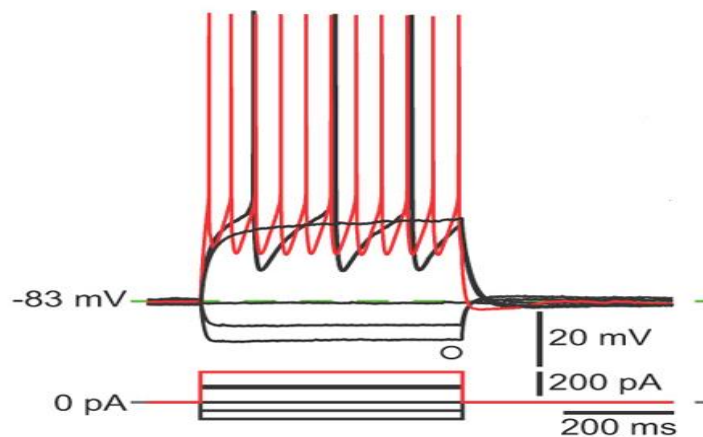


Figure 1.10: Electrophysiological properties of NPY–NGF interneurons.

The NPY–NGF responses to different depolarizing and hyperpolarizing currents show that the NPY–NGF interneuron are characterized by hyperpolarized membrane potential and low input resistance. Adapted from (O. Ibáñez-Sandoval et al., 2011).

NGF interneurons have large highly dense axonal branches which suggests that NGF form several synaptic connections with the surrounding. The recorded synaptic currents showed that the NPY–NGF interneurons exert a powerful feedforward inhibitory effect on MSNs (Osvaldo Ibáñez-Sandoval et al., 2011).

1. 3.2.4 Fast spiking interneurons (FSIs):

FS interneurons are also known as parvalbumin-positive neurons. They are more distributed in the dorsal striatum. Electrophysiologically, FSIs are characterized by hyperpolarized resting potential, low input resistance, short duration action potential and high-frequency

firing hence named FS interneurons (Figure 1.11). There are many gap junctions that are present between FS which increase their level of activity (Kawaguchi, 1993). FSIs mainly target MSNs.

FSIs are modulated through excitatory inputs from cortex (Lapper, Smith, Sadikot, Parent, & Bolam, 1992) and thalamus (Sidibe & Smith, 1999). In contrast, FSIs receive inhibitory innervations from other striatal interneurons (Chang & Kita, 1992), and some neurons in the globus pallidus (Bevan et al., 1998).

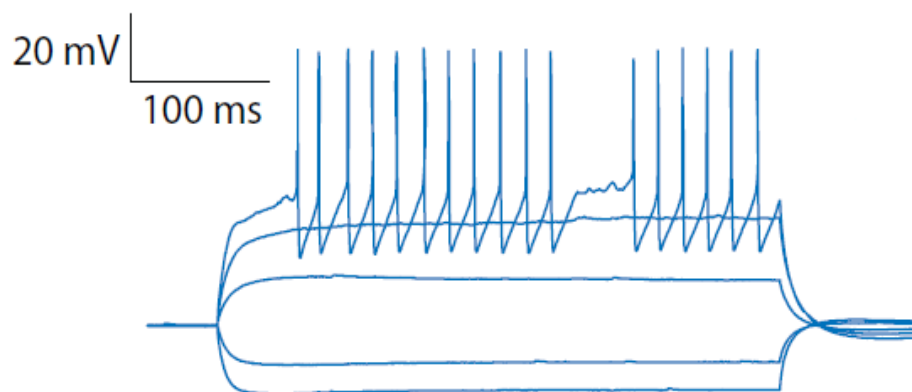


Figure 1.11: Electrophysiological properties of FS interneurons

The FSIs responses to different depolarizing and hyperpolarizing currents show that FSI show rapid firing pattern with low input. Adapted from (Kreitzer, 2009).

Regarding neuromodulation of FSIs, Dopamine excites FSIs via activation of D5 receptor activation and inhibition of D2 receptors on the GABAergic afferents innervating FSIs (Bracci, Centonze, Bernardi, & Calabresi, 2002). Furthermore, ACh stimulate the FSIs by activation of FS nicotinic receptors (F. M. Zhou et al., 2002).

1. 3.2.5 Calretinin Interneurons

Calretinin (CR)-expressing interneurons are medium sized neurons which constitute about 0.8% of neurons in the rodent striatum (V. V. Rymar, R. Sasseville, K. C. Luk, & A. F. Sadikot, 2004). Interestingly, the percentage of CR⁺ neurons is higher in primates including

humans (Wu & Parent, 2000). We do not have a lot of information about neurophysiology of the calretinin interneurons as there is no recording of these cells in vitro, and no way to identify them from in vivo recordings (H. Steiner & K. Y. Tseng, 2010).

1.4 Highlights on some key neurotransmitters involved in modulation of striatal function :

There are many neurotransmitters that modulate the striatal function; however, we will cast the light on the neurotransmitters related to this research which include: acetylcholine, opiate peptides and nitric oxide.

1.4.1 Acetylcholine

ACh pathways have become interesting to study within the context of reward as these pathways interact with the main brain areas regions involved in reward processing.

Sources of acetylcholine and cholinergic receptors

In the brain, the cholinergic system consists of two main parts: the interneuronal system in which interneurons are the source of acetylcholine; and the projection neuronal system which is composed of many nuclei in the forebrain. These nuclei provide a source of ACh through their axons that extend between different brain regions. The striatum is richly innervated by the cholinergic system that suggests that ACh exert a big influence on the basal ganglia function (Toshihiko Aosaki et al., 2010).

Cholinergic receptors are classified into two main categories: the nicotinic and the muscarinic receptors. Muscarinic receptors are subdivided into M1-like receptors (M1, M3, and M5) and M2-like receptors (M2 and M4). The primary effect of M1-like receptors is depolarization and increased neuronal excitability via inhibition of several K^+ currents (Benarroch, 2012). The M2-like receptors main effects are presynaptic inhibition of Ca^{+2} channels, involved in exocytosis, and postsynaptic inhibition via activation of K^+ channels. These receptors act as inhibitory autoreceptors in the cholinergic interneurons and

presynaptic inhibitory receptor in glutamatergic corticostriatal and dopaminergic nigrostriatal terminals (Benarroch, 2012).

On the other hand, the nicotinic receptors (nAChR) are ligand-gated channels that elicit fast excitatory effects both presynaptically and postsynaptically (Benarroch, 2012). The nicotinic receptors (nAChRs) can be classified into three main types based on their physiology and pharmacology: the muscle nAChRs that are present at the neuromuscular junction, and two types of neuronal nAChRs that are present in the CNS (F. M. Zhou et al., 2002). The wide distribution of cholinergic nicotinic receptors has been associated with many effects of ACh on arousal, fatigue, sleep, attention and central processing of pain (Dani, Ji, & Zhou, 2001).

Role of Acetylcholine in the striatum

The cholinergic receptors are widely distributed in the striatum. The excitatory M1 and the inhibitory M4 receptors are expressed in the MSNs. Moreover, the inhibitory M2/M4 and the excitatory nAChRs are expressed in presynaptic glutamatergic and dopaminergic terminals (Oldenburg & Ding, 2011).

Electrophysiological recordings in the striatum showed that GABAergic interneurons are depolarized by nicotinic agonists that increase their action potential dependent GABA release (Koós & Tepper, 2002). Furthermore, the cholinergic activity was found to play an essential role in regulation of DA release (F.-M. Zhou, Liang, & Dani, 2001).

Despite of the tonic release of ACh in the striatum, the widely distributed acetylcholinesterase (Rymar, Sasseville, Luk, & Sadikot, 2004) rapidly degrades ACh. This minimize the desensitization of nAChRs (TOSHIHIKO Aosaki et al., 1995; F. M. Zhou et al., 2002).

The primary effect of ACh on MSNs is depolarization via M1 receptors, which inhibit the K^+ currents that maintain these neurons in a silent ("down") state. This facilitation occurs primarily on MSNs of the indirect pathway (Shen et al., 2007). M1 receptor activation also inhibits L-type channels which prevent influx of Ca^{+2} that activates K^+ channels responsible for afterhyperpolarization. Postsynaptic M1 receptors also modulate NMDA receptor-mediated responses, thus facilitating the "up" state of MSNs (Calabresi, Centonze, Gubellini,

Pisani, & Bernardi, 2000). In contrast, activation of postsynaptic M4 receptors results in inhibition of MSNs, particularly in those of the direct pathway (Oldenburg & Ding, 2011).

Moreover, ACh indirectly affects MSNs via presynaptic modulation of neurotransmitter release from cortical, nigral, and local afferents. ACh, acting via presynaptic M2/M4 receptors, elicits a tonic inhibition of corticostriatal glutamatergic drive on MSNs. Whereas tonically released ACh, via M2/M4 receptors, inhibits dopamine release, ACh can also trigger dopamine release via activation of presynaptic nAChRs at nigrostriatal terminals (Narushima et al., 2007; Oldenburg & Ding, 2011; Z. Wang et al., 2006).

ACh also affects GABA release from terminals of fast spiking inhibitory interneurons. It was reported that ACh can activate nicotinic receptors on FSIs leading to their depolarization with increased GABA release. On the other hand, ACh can activate the presynaptic M4 muscarinic receptors in FSIs will be activated leading to decreased GABA release. So the dual effect of ACh on the FSIs act on different time scale to modify the striatal output (Koós & Tepper, 2002; F. M. Zhou et al., 2002). The following table summarize the modulation of ACh of striatal function.

Table 1.2: ACh modulation of striatal function

	M1-like (Gq/11)	M2-like (M2/M4)(Gi/o)	Nicotinic $\alpha 4\beta 2$
Effect on MSNs	Excitatory (primarily on indirect pathway)	Inhibitory(primarily on the direct pathway)	Not expressed in MSNs
Effect on corticostriatal afferent	No	Presynaptic inhibition of glutamate release	Triggers glutamate release
Effect on nigrostriatal afferent	No	Presynaptic inhibition of DA release	Fast release of DA
Effect on cholinergic neurons	No	Inhibitory autoreceptors	Not expressed
Effect on fast spiking interneurons	No	Inhibits GABA release	Increase GABA release.
Effect on NPY-somatostatin neurons	Increase GABA release		

1.4.2 Opioid peptides

Opioid peptides and opioid receptors

The opioid receptors can be classified into three main groups: μ , δ and κ types. There is also evidence that subtypes of these receptors might exist. These receptors represent targets for different opioid peptides. These opioid receptors act intracellularly through coupling with guanine nucleotide binding proteins (G_i or G_o) to inhibit adenylate cyclase (Bruijnzeel, 2009).

Furthermore, there are three main types of opioid peptides: β -endorphin, enkephalins and dynorphins that activate μ , δ and κ respectively. It was found that β -endorphin stimulates brain reward function. Moreover, β -endorphin has a potent analgesic effect (Amalric, Cline, Martinez, Bloom, & Koob, 1987; L. F. Tseng, Loh, & Li, 1976; Van Ree, Smyth, & Colpaert, 1979). Moreover, enkephalins are suggested to enhance reward processing in the brain and to mediate pro-inflammatory phase during an immunoresponse (Ignatov, Kovalenko, Andreev, & Titov, 1981; Phillips & LePiane, 1982; Salzet & Tasiemski, 2001). Finally, dynorphins are derived from a precursor known as prodynorphin. It was found that prodynorphin is expressed in the ventral and dorsal striatum (Di Benedetto et al., 2004). A summary of opioid receptors and their ligands is presented in the following table.

Table 1.3: Opioid receptors and ligands

Receptor type	Endogenous Ligands	Exogenous Ligands	Antagonists	
			Non-selective	Selective
μ	β -Endorphin	Morphine DAMGO	Naloxone Naltrexone Nalmefene	CTOP
δ	Enkephalins	DPDPE Deltorphin		SDM25N hydrochloride
κ	Dynorphins	U50488H U69593 E 2078		Nor-binal- torphimine (nor-BNI)

Opioid peptides act either presynaptically or postsynaptically. Postsynaptically, opioids usually inhibit neurons, while, the presynaptic actions of opioids mainly inhibit the release of the neurotransmitters. The effect of an opioid in any brain area will depend on its action at the presynaptic sites on both excitatory and inhibitory neurons, plus its postsynaptic effects (Reisine & Bell, 1993).

Role of opioid system in the striatum

In the striatum, opioid peptides were found as one of the important neurotransmitters that share in modulation of striatal function. The opioid peptide dynorphin A was found in direct pathway MSNs, whilst enkephalin was found in the indirect pathway (Lee, Kaneko, Taki, & Mizuno, 1997). Also, studies on the striatum proved that it contains the three subfamilies of opioid receptors (μ , δ , κ). In situ localization studies indicated that μ , δ and κ receptors present in both striatopallidal and striatonigral neurons. μ receptors are specially located in the striatal patches, while δ receptors were found on the surface of cholinergic interneurons (H. Wang & Pickel, 2001).

Some studies reported that κ receptors in the striatum are synthesized in the ventral tegmental area (VTA) and substantia nigra (SN). Then κ receptors are transported to the striatum. Moreover, κ receptors in striatum modulate the dopamine release (Britt & McGehee, 2008). Furthermore, high levels of κ receptors RNA were found in the shell of nucleus accumbens and the dorsal striatum (Meng et al., 1993; Simonin et al., 1995; Yasuda et al., 1993; F. M. Zhou et al., 2002).

The opioid system in the striatum plays a crucial role in reward processing through mediating hedonic evaluation of natural rewards. Although, many studies focused on the nucleus accumbens role in processing of reward, evidences indicate that the dorsal striatum plays a vital role in brain reward system (P. Apicella, Ljungberg, Scarnati, & Schultz, 1991).

The opioidergic modulation of striatal function is still poorly understood. Jiang and North, 1992 showed that opioids presynaptic inhibit the corticostriate excitatory synaptic inputs. In their study, opioids μ - and δ -receptors selective agonists inhibited the excitatory postsynaptic potential presynaptically, while, δ -selective agonists decreased the inhibitory synaptic potential. They also reported that δ -selective agonists inhibit a small subpopulation of striatal cells (most probably cholinergic interneurons) (Z. G. Jiang & R. A. North, 1992).

In another group of studies, it was found that stimulation of μ and δ receptors present on cholinergic interneurons in the NAcc, inhibits the release of ACh from these neurons, thus decreasing the ACh levels (Dourmap, Clero, & Costentin, 1997; Lapchak, Araujo, & Collier, 1989).

From another prospective, behavioural studies proved that injections of μ or δ but not κ agonists stimulate feeding in the NAc (Baldo & Kelley, 2007; Bodnar, 2004). Moreover, some studies reported the presence of a hedonic hotspot in the shell of the NAc in which a dense presence of μ receptors. Stimulation of μ receptors in the hedonic hotspot increases the food "liking". On the other hand, stimulation of the surrounding region of this hotspot increase the food intake with no effect on the food "liking" (Berridge, 2000; Peciña, Smith, & Berridge, 2006). Moreover, some studies showed NAc mediates the reinforcing properties of opiates (David & Cazala, 1994). On the other hand, intra-NAc microinjections of the κ receptor agonist produced aversive effects in lab animals (Bals-Kubik, Ableitner, Herz, & Shippenberg, 1993). Interestingly, Braida et al. (2008) reported that very small doses of κ receptor agonists can induce some reinforcement while high doses of κ receptor agonists cause negative mood states. Moreover, it was found that, small doses of κ receptor agonists decrease morphine rewarding properties in rodents (Bolanos, Garmsen, Clair, & McDougall, 1996). Moreover, κ receptor antagonists increase the dopamine release in the striatum (Beardsley, Howard, Shelton, & Carroll, 2005).

It worth mentioning that opioid system in the striatum not only shares in opiate reinforcement, but also in the reinforcing effects of non-opioid drugs which include cocaine, nicotine, and alcohol. These non-opioid drugs may lead to an increase of the local endogenous opioids (Le Merrer, Becker, Befort, & Kieffer, 2009). Moreover, opioids play an important role in pain sensation. It was proposed that patients with lesions in the striatum experience a decrease in pain sensitivity (Starr et al., 2011).

1.4.3 Nitric oxide

Nitric oxide (NO) is a gaseous neurotransmitter that plays a crucial role in different physiological and pathological conditions in the peripheral and central nervous system (Boehning & Snyder, 2003; Bredt, 2003; Garthwaite, 2008). In the nervous system, NO is synthesized by the neuronal nitric oxide synthase (nNOS) (Alderton, Cooper, & Knowles, 2001; Garthwaite, 2008). This enzyme is expressed in moderate levels in the basal ganglia nuclei (Bredt & Snyder, 1990; Vincent, 1994). LTSIs are the only known source of NO in the

striatum (Kawaguchi, 1997; Yoshiyuki Kubota, Mikawa, & Kawaguchi, 1993; J. M. Tepper & Bolam, 2004).

There are many intracellular signal transduction pathways for NO transmission inside neurons. The most well-known signal transduction pathway is through activation of the soluble guanylyl cyclase (sGC) signaling cascade (Garthwaite, 2008; Murad, 2006). Besides the cGMP cascade, some studies demonstrated that NO can act intracellularly through non-cGMP mechanisms such as facilitation of calcium release from intracellular calcium stores in striatal neurons (HORN et al., 2002; Meini et al., 2000). Moreover, NO may alter NMDA and GABA_A receptor function, and potassium, calcium, chloride, and non-selective ion channels via direct and indirect mechanisms (Ahern, Klyachko, & Jackson, 2002).

Electrophysiological studies showed that NO interneurons are activated by corticostriatal afferents (Berretta, Parthasarathy, & Graybiel, 1997; Kawaguchi, 1993). It was found that these interneurons express NMDA, AMPA and metabotropic glutamate receptors (Gracy & Pickel, 1997; Kawaguchi, 1997; Kawaguchi et al., 1995) and receive asymmetric glutamatergic inputs from the frontal cortex (Salin, Kerkerian-Le Goff, & Epelbaum, 1990; Vuillet, Kerkerian, Kachidian, Bosler, & Nieoullon, 1989).

In situ hybridization studies done in rodents showed that NO interneurons express low levels of D1 dopamine receptor mRNA (Le Moine, Normand, & Bloch, 1991). They also express D5 receptor protein (Diego Centonze et al., 2003; Rivera et al., 2002). Accordingly, electrical and chemical stimulation of the SN and systemic administration of the D1/5 receptor agonists increased striatal NO efflux. These facilitatory effects decreased by administration of D2 receptor agonist (Liu, Liu, Gao, & Li, 2005; Sammut, Bray, & West, 2007; Sammut et al., 2006). Thus dopamine can modulate NO synthesis and NO interneuron activity via direct stimulation of D1-and D2-like receptors.

Several *in vivo* studies on rats showed that striatal levels of nitrite (Ohta et al., 1994), cGMP (Globus, Prado, & Busto, 1995) and L-citrulline (Ohta et al., 1994) are maintained in a steady-state which is sensitive to changes in NOS activity. These levels “tonic” NO and cGMP play an important signalling role in the striatum. On the other hand, stimulation of NO interneurons is associated with “phasic” release of NO which can be excitatory or inhibitory effects to other striatal neurons (Galati et al., 2008).

Role of NO in the striatum

It was proved that endogenous and exogenous NO facilitates the release of several neurotransmitters in the striatum including glutamate, DA, and acetylcholine (ACh) (Janos P Kiss, 2000; Helmut Prast & Philippu, 2001; West, Galloway, & Grace, 2002).

NO can act to facilitate glutamate transmission through different mechanisms that include: (1) inhibition of glutamate reuptake (Lonart & Johnson, 1994; Sakire Pogun, Valina Dawson, & Michael J Kuhar, 1994; Taskiran, Kutay, & Pogun, 2003) and (2) facilitation of glutamate release via a calcium-independent mechanism (Meffert, Premack, & Schulman, 1994). Additionally, intrastriatal cGMP infusion increases extracellular glutamate via an unknown mechanism (Guevara-Guzman, Emson, & Kendrick, 1994; M. Kraus & Prast, 2002). Thus, these studies indicate that NO facilitation of glutamate transmission is a widespread and highly conserved mechanism involved in the nitroergic modulation of synaptic transmission.

Furthermore, it was reported that NO facilitates DA efflux *in vitro* (Chaparro-Huerta, Beas-Zárate, Guerrero, & Feria-Velasco, 1997; Liang & Kaufman, 1998; Zhu & Luo, 1992) and *in vivo* (Spatz et al., 1995; Strasser, McCarron, Ishii, Stanimirovic, & Spatz, 1994; A. R. West & M. P. Galloway, 1997; West et al., 2002). This facilitation occurs either through a glutamate receptor-dependent mechanism (Nakahara, Yokoo, Yoshida, Tanaka, & Shigemori, 1994; Rocchitta et al., 2004; A. West & M. Galloway, 1997; West & Galloway, 1996; West et al., 2002) or inhibition of the DA transporter (János P Kiss, Zsilla, & Vizi, 2004; Sakire Pogun et al., 1994).

Studies done on both awake and anesthetized rats demonstrated that low and moderate concentrations of NO generators (diethylamine/NO, SNAP, or 3-morpholinopropanesulfonamide (SIN-1)) facilitated the release of ACh in the nucleus accumbens (M. M. Kraus & Prast, 2001; H Prast, Fischer, Werner, Werner-Felmayer, & Philippu, 1995; H Prast, Tran, Fischer, & Philippu, 1998). At higher concentrations, local infusion of diethylamine/NO decreased the release rate of ACh via a GABA_A receptor-dependent mechanism (H Prast et al., 1998). Further work showed that the sGC-cGMP signalling pathway is critically involved in the modulation of ACh efflux by NO (Guevara-Guzman et al., 1994; M. Kraus & Prast, 2002; H Prast et al., 1998). Moreover, it was reported that NO increases the firing of cholinergic interneurons in the striatum (Centonze et al., 2001).

Studies on parkinsonian patients suggest that NO system plays a key role in the pathophysiology of Parkinson disease (Blanchet et al., 1999; Bredt & Snyder, 1990; Gupta et al., 2014; Pavon, Martín, Mendiola, & Moratalla, 2006). Some studies reported an increase in the concentration of NO metabolites (Molina et al., 1996; Qureshi et al., 1995), nNOS overexpression in neutrophils (Gatto et al., 2000), and cGMP concentrations in the plasma and cerebrospinal fluid (Brodacki et al., 2011; Chalimoniuk & Stêpień, 2004; Navarro et al., 1998). On the other hand, post-mortem studies in PD patients reported a decrease in nNOS mRNA expression (Eve et al., 1998). Moreover, a decrease in the cGMP concentrations in the plasma and CSF was reported (Belmaker et al., 1978).

In animal models, contradicted findings were also reported. 6-OHDA-lesioned rats demonstrated an increase in the nNOS protein in the striatum (F. E. Padovan-Neto, Echeverry, Chiavegatto, & Del-Bel, 2011). Similarly, rotenone (He et al., 2003) or manganese chloride (Ponzoni, Guimarães, Del Bel, & Garcia-Cairasco, 2000) administration to rats significantly increased the number of NADPH-d/nNOS-positive cells/fibres and the NOS activity in the striatum.

Moreover, it was reported that administration of the ODQ (sGC inhibitor) to 6-OHDA-lesioned rats attenuated the increase of cGMP levels in the striatum, the excessive abnormal firing of striatal medium spiny neurons and the increased metabolic activity of the subthalamic nucleus (STN). Furthermore, ODQ decreased akinesia in 6-OHDA-lesioned rats and in MPTP-treated mice (Tseng et al., 2011).

On the other hand, a decrease in the level of nNOS protein and the number of nNOS-immuno-positive intrastriatal fibres in the striatum of 6-OHDA-lesioned rats was reported (Sancesario et al., 2004). Furthermore, it was reported that nNOS-immunoreactive cells were unchanged in MPTP-treated mice (Muramatsu et al., 2002).

Better understanding of the role of NO-cGMP pathway in PD will help in the development of new therapies aiming to restore motor function.

However, NOS inhibitors represent a newly potential pharmacological approach for counteracting L-DOPA induced dyskinesia (LIDs). Many behavioural studies demonstrated that NOS inhibitors can counteract LIDs. Moreover, Anti-dyskinetic effects of NOS inhibitors have been well characterized in preclinical investigations in rodents (Del-Bel, Eduardo Padovan-Neto, Raisman-Vozari, & Lazzarini, 2011; Novaretti, Padovan-Neto, Tumas, Da-Silva, & Del Bel, 2010; F. Padovan-Neto, Echeverry, Tumas, & Del-Bel, 2009; F. E.

Padovan-Neto et al., 2011; F. E. Padovan-Neto et al., 2013; Takuma et al., 2012) and non-human primate (Yuste et al., 2012). These processes in human remain to be proved.

1.5 Aim of this study

The primary aim of this project is to explore the role of the LTSIs in modification of the striatal microcircuitry. The LTSIs express many important neurotransmitters (i.e. GABA, somatostatin, NPY, and NO) in the striatum (Kawaguchi et al., 1995). Moreover, LTSIs are one of the tonically active neurons of the striatum.

Previously, studies on striatal LTSIs were limited due to their small numbers and their undistinguishable morphology under microscopy (Kawaguchi, 1993; Y. Kubota & Kawaguchi, 2000). This problem was solved through developing transgenic mice in which a humanized Renilla GFP (hrGFP) is expressed under the control of NPY promoter can be used for easy identification of the LTSIs under fluorescent microscope (J. G. Partridge et al., 2009).

The mutual interaction between the LTSIs and the cholinergic interneurons in the striatum has not been studied before in details. It was reported that acetylcholine can stimulate LTSIs through nicotinic activation (Luo, Janssen, Partridge, & Vicini, 2013). Being both tonically active make the understanding of how these two neurons tune the action of each other an interesting point of research. Furthermore, the optogenetic revolution in the recent years allowed the targeted stimulation of neurons that provide more physiological alternative to the pharmacological application. Using transgenic mice strain in which ChR2 is only expressed on somatostatin positive cells, we were able to study the effect of activation of LTSIs on cholinergic interneurons.

Moreover, we further sought to investigate the role of opiate peptides in modulation of the activity of the LTSIs directly and indirectly through modification of the activity of the cholinergic interneurons and the GABAergic transmission on LTSIs. Opioids are present in the striatum. Dynorphin is expressed by the striatonigral MSNs, while enkephalin is expressed by the striatopallidal MSNs (Charles R. Gerfen, 1992; Gertler, Chan, & Surmeier, 2008). These opiate peptides play an essential role in reward and drug abuse. Linking the effect of opiate peptides on LTSIs to the mutual interaction between LTSIs and cholinergic

interneurons is expected to deepen our understanding of the microcircuitry inside the striatum. Figure (1.12) details the primary circuits to be investigated.

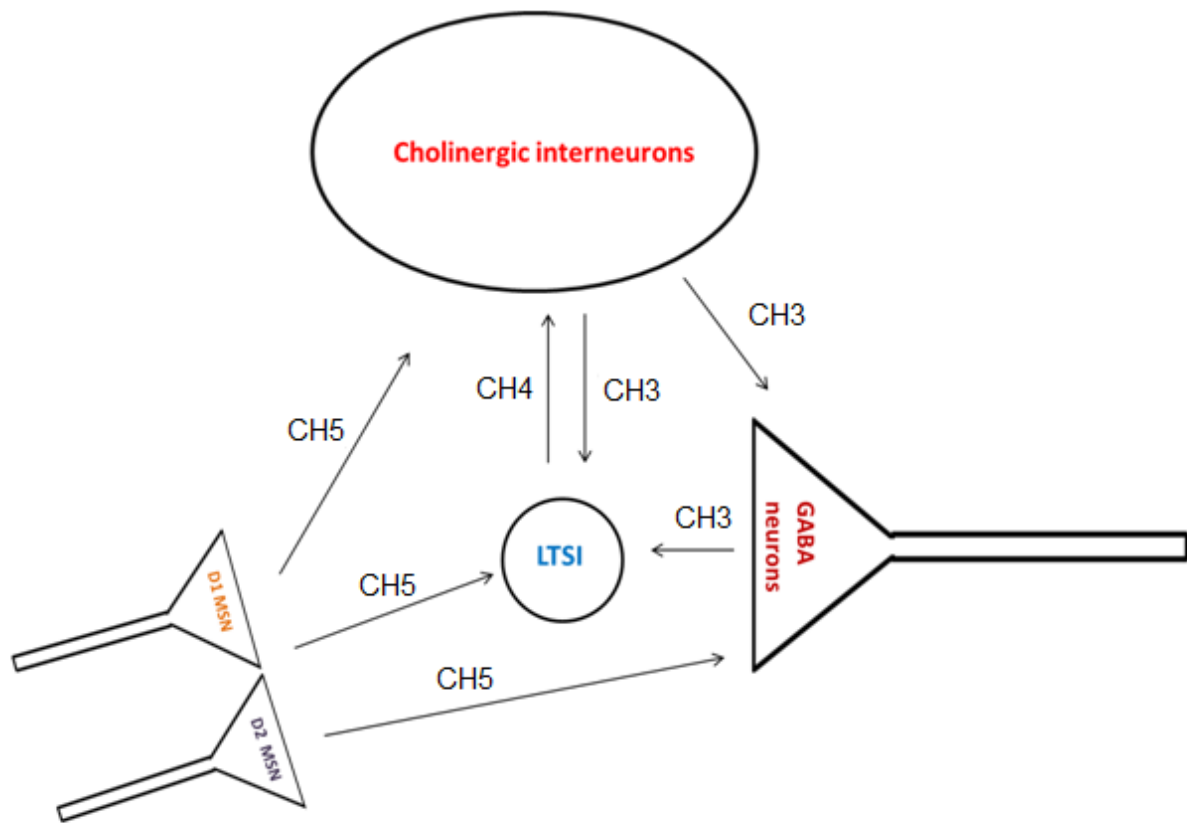


Figure (1.12): Principle circuits of investigation.

The aims of this research project include: (1) investigation of the cholinergic modulation of LTSIs (directly and on GABAergic transmission on LTSIs) (chapter 3; CH3) (2) investigation of nitrenergic modulation of cholinergic interneurons (chapter 4; CH4), and (3) investigation of the effect of opiates peptides on and cholinergic interneurons, LTSIs and GABA transmission on LTSIs (chapter 5; CH5).

Chapter Two: Materials & Methods

2.1 Animals:

The experiments were carried out using two colonies of transgenic mice. The first colony is a hemizygous transgenic NPY-GFP mice colony in which a humanized Renilla Green Fluorescent Protein (hrGFP, Stratagene) is expressed under control of neuropeptide Y (NPY) promoter. These mice were obtained through insertion of GFP (hrGFP, Stratagene) sequence into the start of the transitional site of the neuropeptide Y (NPY) gene (Stock 006417, Jackson Laboratory, USA). This colony was bred by crossing between the hemizygous BAC-NPY transgenic males mice and wild-type (CD57) females. In these animals NPY positive interneurons in brain are marked with GFP. In the striatum, NPY positive cells include LTSIs and neurogliaform interneurons. The age of the NPY-GFP mice ranged between 16 and 32 days.

The second transgenic mice colony is a heterozygous transgenic ChR2-EYFP-SOM-IRES-Cre mice colony. These mice obtained by crossing between homozygous SOM-IRES-Cre mice (Stock 013044, Jackson Laboratory, USA) and a homozygous ChR2 (H134R)-EYFP mice (Stock 024109, Jackson Laboratory, USA). The SOM-IRES-Cre (or Sst-IRES-Cre) mouse genome is characterized by the presence of Cre recombinase in the 3' untranslated region (after the translational termination site) of the somatostatin locus. In this case, the endogenous somatostatin promoter will direct Cre expression in these mice. Thus, Cre recombinase activity will be observed in somatostatin positive neurons.

On the other hand, the ChR2 (H134R)-EYFP mice are characterized by the expression of ChR2 (H134R)-EYFP protein. The ChR2 (H134R) is activated by blue light allowing Na⁺ ions entry to the cell. The Na⁺ entry leads to depolarization and action potentials (Madisen et al., 2012). The resulting offspring of breeding of homozygous SOM-IRES-Cre mice and homozygous ChR2 (H134R)-EYFP mice are characterized by the expression of the ChR2 (H134R)-EYFP fusion protein in the somatostatin positive neurons. When ChR2 (H134R)-SOM expressing cells are exposed to light (450 - 490 nm), these cells will be firing due to the reversible photo-stimulation of ChR2 protein. The age of these mice ranged from 21 and 35 days.

All animals were bred and housed in the Biological Services Facility, University of Sheffield.

2. 2 Slice preparation:

For all experiments, mice (P16-32 for NPY-GFP & P21-35 for ChR2-EYFP-SOM-IRES-Cre) of both sexes were used for optimal neuronal viability and identification. Mice were deeply anesthetized with inhaled isoflurane then perfused transcardially with 5–10 ml of oxygenated ice cold modified HEPES artificial CSF (aCSF) solution containing (in mM) : 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4 , 30 NaHCO_3 , 20 HEPES, 25 Glucose, 5 sodium ascorbate, 2 Thiourea, 3 sodium pyruvate, 10 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at pH7.4. The animals were subsequently killed by decapitation.

After the perfusion, we quickly removed the brain from the skull and dissected on ice. Using a vibroslicer (Camden instruments), the sagittal brain slices (250 μm thick) were prepared. Slices were immediately transferred to a storage chamber which contains the modified HEPES ACSF that is continuously bubbled with a carbogen mixture of 95% O_2 and 5% CO_2 gas. The slices were kept at 35 °C for 15 minutes then temperature was lowered to 24 °C till the end of experiment. The slices were left for at least 1 hour to equilibrate before electrophysiological recording.

During recording, slices were placed in a recording chamber. Slices were continuously superfused with oxygenated standard aCSF containing (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4 , 24 NaHCO_3 , 5 HEPES, 12.5 Glucose, 2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ at pH7.4 (flow rate 1.5 -2ml/min) at room temperature.

2. 3 Slice visualization and optogenetic stimulation

Slices were visualized using an infrared/differential interface contrast microscopy by a 40x water-immersion objective. For special visualization of the NPY-positive cells in the NPY-GFP mice, we used epifluorescence with a standard GFP filter coupled with a mercury lamp (Olympus U-RFL-T)

For optogenetic visualization & stimulation we used a High power blue LED driver (DC2100, ThorLabs). Visualization of the cells was carried out using constant current mode which provides a constant non-modulated LED current. The stimulation was carried out using the external mode which allows controlling of the DC2100 by an external signal. 2, 5 & 10 seconds pulses were applied for stimulation of slices obtained from the ChR2-EYFP-SOM-IRES-Cre positive mice.

2.4 Electrophysiological recording

GFP-positive neurons in striatum are LTSIs and NGFIs. LTSIs were initially identified by being less bright than NGF with epifluorescence. Then, their identity was confirmed by examining membrane potential characteristics during recording (Kawaguchi, 1993). Cholinergic interneurons were identified by their big size compared to the other striatal neurons. Their identity was confirmed by examining distinctive electrophysiological properties.

The current-clamp recordings were carried out in bridge mode using NPI BA-1S bridge amplifier. Patch pipettes (3-6M Ω) were prepared by pulling borosilicate glass tubes with a PC-10 puller (Narishige group).

In the whole cell and the cell attached recordings, the patch pipettes were filled with an intracellular solution containing in (mM): 120 K- gluconate, 20 KCl, 2 MgCl₂, 12 HEPES, 0.4 Na-GTP and 4 Na⁺-ATP, 0.04 EGTA. The intracellular solution was adjusted to pH 7.3 with KOH. Before each experiment, both the offset and the capacitance of the pipette tip were compensated out.

For whole cell recordings, the tip of the patching pipettes was placed on the soma of the neuron then by applying suction a seal with resistance of at least 1G Ω was formed. After forming the seal, whole-cell configuration was attained by applying another gentle brief suction.

Cell-attached recordings were obtained with similar procedure but the membrane was not ruptured. In these conditions, we were able to detect spontaneous spikes of LTSIs as rapid biphasic deflections of the recorded potential.

For perforated whole-cell recordings, 3 mg of amphotericin B were dissolved in 50 μ l DMSO to get 60 mg/ml stock solution. Then 20 μ l of the stock solution were added to 5 ml of intracellular solution to reach a final concentration of 240 μ g/ml. The tips of the patching pipettes were filled with amphotericin B-free intracellular solution to help the seal formation. Once a gigaohm seal is obtained, the cell is left for 10-15 minutes to allow the amphotericin B pores to be formed. The perforation was usually complete within 15 minutes from the seal formation. The full perforation was confirmed the action potential was >50 mV and the electrode resistance was < 50 M Ω . Amphotericin B is known to be a light sensitive chemical, so the whole procedure was carried in darkness to minimize the exposure of the drug to the light.

During testing the GABAergic IPSPs, we used high chloride intracellular solution in which equimolar KCl substituted K-Gluconate. GABA_A receptors are ligand-gated ion channel receptors. Once GABA binds to the receptor, it will lead to opening of ion channel that allow the in movement of Cl⁻ ions from extracellular fluid to the intracellular fluid. Using high Cl⁻ intracellular solution set the Cl⁻ reversal potential near to zero. This will increase the amplitude of the IPSPs at the negative membrane potentials that are suitable for whole-cell recording. Moreover, High Cl⁻ intracellular solution proved to increase the decay time of GABAergic IPSPs. Thus the GABA signals will be amplified leading to easier study of these events (Gallagher, Higashi, & Nishi, 1978; Houston et al., 2009).

For the evoked IPSPs we applied an electrical stimulation by placing one pole in a glass electrode (0 M Ω) filled with aCSF near the recorded cell in order to stimulate the local GABAergic fibers. The second pole was connected to the ground electrode. The intensity of the electrical stimulation was around 100 μ A – 1000 μ A, the duration ranged between 100 μ s- 1000 μ s and the interval between the two stimulations was 200 ms. During the recording of the spontaneous and evoked IPSPs, the cell membrane potential was kept constant at -80 mV through the whole experiment by injecting negative current into the cell. Whenever, the applied drug depolarized or hyperpolarized the cell, the amount of the injected negative current was adjusted to keep the membrane potential at -80 mV.

2. 4 Drugs

All drugs were prepared from dissolved stock solutions. Drugs were applied by adding the appropriate amount to the superfusing solution. Drugs usually reached the slice within 4-5 minutes from application through a gravity system.

The following drugs were used: atropine sulfate (20 μ M; muscarinic acetylcholine receptor antagonist, Sigma) , nicotine (1 μ M; to cause desensitization of nicotinic receptors (Britt & McGehee, 2008), Sigma) , mecamylamine hydrochloride (10 μ M ; non-competitive nicotinic acetylcholine receptor antagonist, Cambridge Bioscience) . Moreover, picrotoxin (100 μ M; GABA_A receptor antagonist, Tocris) & (-)-bicuculline methiodide (25 μ M; GABA_A receptor antagonist, Tocris) were used to block GABA_A receptors. D-AP5 (10 μ M; competitive NMDA antagonist, Tocris) and NBQX (10 μ M; selective AMPA receptor antagonist, Tocris) were used to block the glutamate receptors by blocking ionotropic glutamate receptors.

For optogenetics experiments we used the following drugs: ODQ (10 μ M; selective inhibitor of NO-sensitive guanylyl cyclase, Tocris), cyclosomatostatin (1 μ M; Non-selective somatostatin (sst) receptor antagonist, Tocris), PD 160170 (20 μ M; selective neuropeptide Y1, Y2 and Y5 receptors ($K_i > 10$ μ M), Tocris) and L-NAME hydrochloride (100 μ M; NO synthase inhibitor, Cayman chemical company).

Moreover, opioid agonists that were used are: DAMGO (1 μ M; selective μ - receptor agonist, Tocris), DPDPE (1 μ M; selective δ - receptor agonist, Tocris), and U50488H (10 μ M; selective κ -receptor agonist, Tocris). While, opioid antagonists used were CTOP (1 μ M; selective μ - receptor antagonist, Tocris), SDM25N hydrochloride (1 μ M; selective δ - receptor agonist, Tocris), GNTI dihydrochloride (1 μ M; potent κ opioid receptor antagonist, Tocris) and naloxone hydrochloride (10 μ M; non selective opioid antagonist, Tocris).

Finally, we used tetrodotoxin citrate (TTX) (1 μ M; selective sodium channel blocker, Tocris) to block the action potentials and Amphotericin B (Sigma) was used as a pore forming agent for perforated patch recordings.

2. 5 Data analysis

Data were acquired using Signal (2.9) software and a micro 1401 data acquisition unit (CED, Cambridge, UK). Off-line data analysis was done with Signal, spike 2, Origin Pro8 (Origin lab), and Microsoft Excel. Figure preparation was done with Adobe Photoshop and Illustrator (Adobe Systems).

We tested the neurons electrophysiological properties in absence of any drug. We used neurons with clear and stable properties i.e. stable resting V_m ; average amplitude of APs; low noise; and constant input resistance. When input resistance altered by more than 25% (not due to drug application), the cell was discarded.

The first 10 minutes after application of any drug were excluded from the analysis of the experiments to ensure that the drug reached the desired concentrations around the tissue.

Spontaneous and evoked GABAergic activity was recorded in the presence of NBQX (10 μ M) and D-APV (10 μ M) to isolate inhibitory synaptic responses through minimizing of the excitatory effect of glutamate by blocking ionotropic glutamate receptors.

2. 6 Statistical analysis

The effect of different drugs was assessed through comparing the firing frequency, inter spike interval (ISI), and membrane potential before and after the application of each ligand. The firing frequency (Hz) was measured by dividing the number of spikes in single frame by the duration of the frame (in seconds). Inter spike interval (ISIs) was defined as the time between the peak of one spike and the peak of the successive one.

In the experiments in which the IPSPs were tested; paired-pulse ratio (PPR) was calculated as 2^{nd} IPSP/ 1^{st} IPSP. Because the PPR ratios are not normally distributed, Mann–Whitney U test was used to test significance of change. Mann–Whitney U test is one of the nonparametric tests of the null hypothesis. Mann-Whitney U test is used for statistical comparison of two samples from the same population against an alternative hypothesis.

In the experiments of the spontaneous GABAergic activity, cumulative frequency and amplitude were calculated in both the control and after drug application.

When testing across a group of neurons, mean responses for each condition in a single neuron were calculated and then normalized against the control mean for that neuron to produce a figure for effect of drug in each state.

The mean value was calculated for the neurons in which significant effects were observed. All results are expressed as mean \pm SD. Student's unpaired *t*-test was used for statistical comparisons (unless otherwise stated). Statistical significance was determined if $p < 0.05$.

Chapter Three: Cholinergic control of LTSl activity

3.1 Abstract

Striatal output is modified by many neurotransmitters released by local interneurons or axons of other projection neurons. Cholinergic interneurons and nitrgergic interneurons represent the only known spontaneously active neurons inside the striatum. The effect of endogenously released acetylcholine in the striatum on LTSIs was not studied before. Using transgenic mice in which NPY-expressing neurons are marked with a green fluorescent protein (GFP), the effects of blocking the cholinergic receptors on LTSI activity was studied. Blocking of cholinergic muscarinic receptor using atropine sulfate (20 μ M) led to activation of the LTSIs in control and in TTX solutions. On the other hand, blocking of cholinergic nicotinic receptors using either mecamylamine hydrochloride (10 μ M) or nicotine (1 μ M) caused inhibition of the LTSIs in control and in the presence of TTX.

When both cholinergic receptor blockers (muscarinic and nicotinic) were applied simultaneously, we observed an inhibition of LTSIs in control solution and stimulation in the presence of TTX. Moreover, the presence of GABAergic tone on LTSIs was investigated and we found that GABA_A blockers caused a significant depolarization of the LTSIs in control and in TTX.

Furthermore, the cholinergic modulation of the GABAergic transmission on LTSIs was investigated. Our data demonstrate inhibition of spontaneous and evoked GABAergic IPSPs by mecamylamine. On the other hand, there was a facilitation of these GABAergic IPSPs with atropine. In presence of both the nicotinic and the muscarinic receptor blockers, GABAergic transmission was facilitated.

It was concluded that acetylcholine modulate the LTSI activity directly and indirectly. The dominant direct effect of the acetylcholine is inhibitory on LTSIs (through muscarinic receptors). While, indirectly the dominant effect of acetylcholine will be stimulatory through inhibition of the GABAergic transmission on LTSIs. This data helps in understanding the effects of two prominent tones (the GABAergic and the cholinergic) on LTSIs activity.

3.2 Introduction

LTSIs and cholinergic interneurons have been reported to be spontaneously active in acute striatal slices. These two types of interneurons also represent the TANs in the striatum (Beatty et al., 2012; Bennett & Wilson, 1999; Dehorter et al., 2009; Gittis, Nelson, Thwin, Palop, & Kreitzer, 2010; Osvaldo Ibáñez-Sandoval et al., 2011; John G Partridge et al., 2009; F. M. Zhou et al., 2002).

Due to characteristic morphological properties, many studies were focused on cholinergic interneurons *in-vitro*. Cholinergic interneurons are considered to be the main source of acetylcholine in the striatum (Graybiel et al., 1994; S. M. Hersch, C. Gutekunst, H. Rees, C. J. Heilman, & A. I. Levey, 1994; Weiner, Levey, & Brann, 1990). Cholinergic interneurons are characterized by large somas and extensive axonal branching (C. Wilson, Chang, & Kitai, 1990), making them well positioned to exert strong cholinergic influence that locally control the dynamics of striatal network.

On the other hand, the studies carried out on LTSIs were limited for a long time due to their small number and absence of distinctive morphological properties. Recently, many strains of transgenic animals in which LTSIs can be easily identified were developed. This gave the opportunity to study the role of LTSIs in modification of striatal network.

The cholinergic modulation of the LTSI activity was not studied in details previously. However, it was reported that acetylcholine can stimulate LTSIs through nicotinic activation (Luo, Janssen, Partridge, & Vicini, 2013). Furthermore, direct application of nicotinic agonists has been reported to generate inward currents or depolarize certain classes of striatal interneurons (De Rover, Lodder, Kits, Schoffemeer, & Brussaard, 2002; English et al., 2012; Koós & Tepper, 2002).

Moreover, activation of cholinergic interneurons has been shown to increase synaptic GABA input to MSNs through modulation of GABAergic interneurons (De Rover et al., 2002; English et al., 2012; Sullivan, Chen, & Morikawa, 2008; Witten et al., 2010). In this study we aimed to study the effects of endogenously released acetylcholine on the LTSI activity.

Furthermore, recently it was reported the presence of tonic GABAergic inhibition in MSNs (Ade, Janssen, Ortinski, & Vicini, 2008; Janssen, Ade, Fu, & Vicini, 2009; Santhakumar,

Jones, & Mody, 2010). These studies have confirmed that tonic GABAergic conductance, mediated by high-affinity GABA_A receptors, regulates MSN excitability. There is evidence that suggests that MSN GABAergic tonic conductance may have cholinergic origin (English et al., 2012; Osvaldo Ibáñez-Sandoval et al., 2011).

Thus we also aimed to investigate the presence of a GABAergic tone affecting the LTSl activity. Moreover, we investigated if cholinergic interneurons play a role in the regulation of the GABAergic transmission on LTSl.

3.3 Results

3.3.1 Electrophysiological identification of striatal low-threshold spiking interneurons

A total 77 BAC-NPY mice (both males & females) aged 25 ± 5 days were used in these experiments. Current-clamp recordings were obtained from 91 striatal GFP-LTSIs neurons; 90 of these recordings were whole-cell and 1 recording was a cell-attached recording.

Examining the slices under the epifluorescence microscopy revealed the presence of GFP-positive cells in the cortex and to a lesser extent in the striatum. GFP positive cells are either LTSIs or NGF. LTSIs differed from NGF cells by having less bright fluorescent (O. Ibáñez-Sandoval et al., 2011) (Figure 3.1.A). They are also characterized by their medium size and few dendritic branches.

During whole cell recordings, LTSIs were identified by their distinctive electrophysiological properties which included (Figure 3.1):

- i. Spontaneous firing activity of LTSIs without any stimulation in 82/82 of LTSIs. Average spontaneous firing frequency was 5.1 ± 1.1 Hz.
- ii. Different patterns of spontaneous firings (regular, irregular and burst firing) with ability to shift spontaneously between them (Beatty et al., 2012) (Figure 3.1B).
- iii. Relatively depolarized membrane potential (Kawaguchi, 1993). Average membrane potential in absence of any injected current was -55 ± 3.2 mV. However, some LTSIs exhibited fluctuations in membrane potential as previously reported (O. Ibáñez-Sandoval et al., 2011).
- iv. Presence of low-threshold spikes which can be seen either in response to depolarizing current injections or as a rebound after injection of a pulse of hyperpolarizing current (J. G. Partridge et al., 2009) (Figure 3.1C).
- v. High input resistance ($> 500\text{M}\Omega$).

In cell-attached recordings, we were able to detect spikes as biphasic deflections of potential. This spikes showed variable patterns of spontaneous firing as in whole-cell recordings (Figure 3.1D).

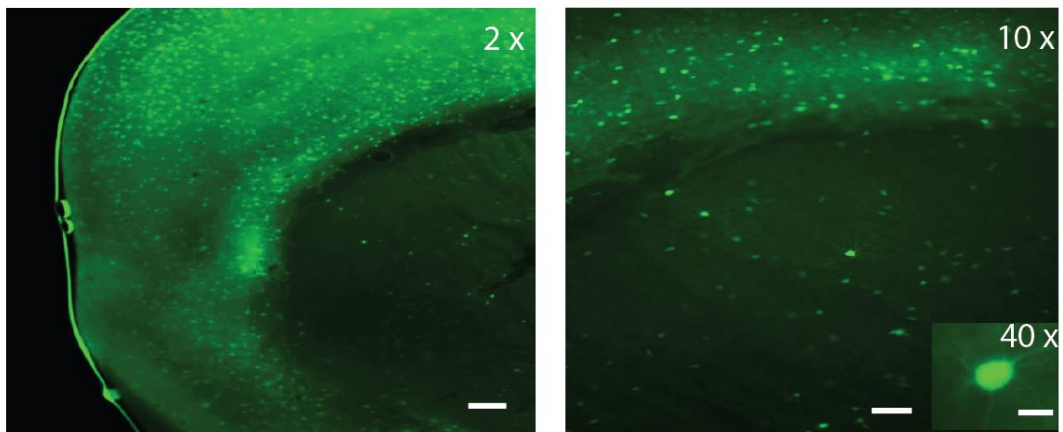
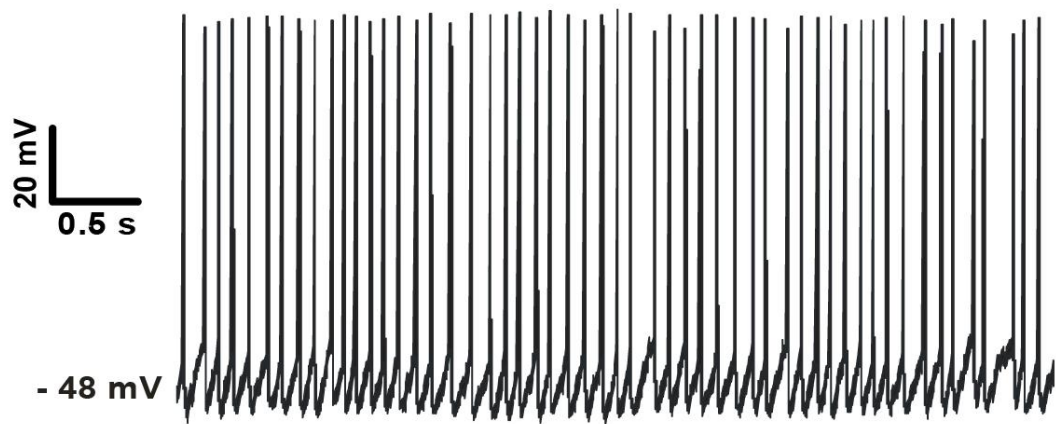
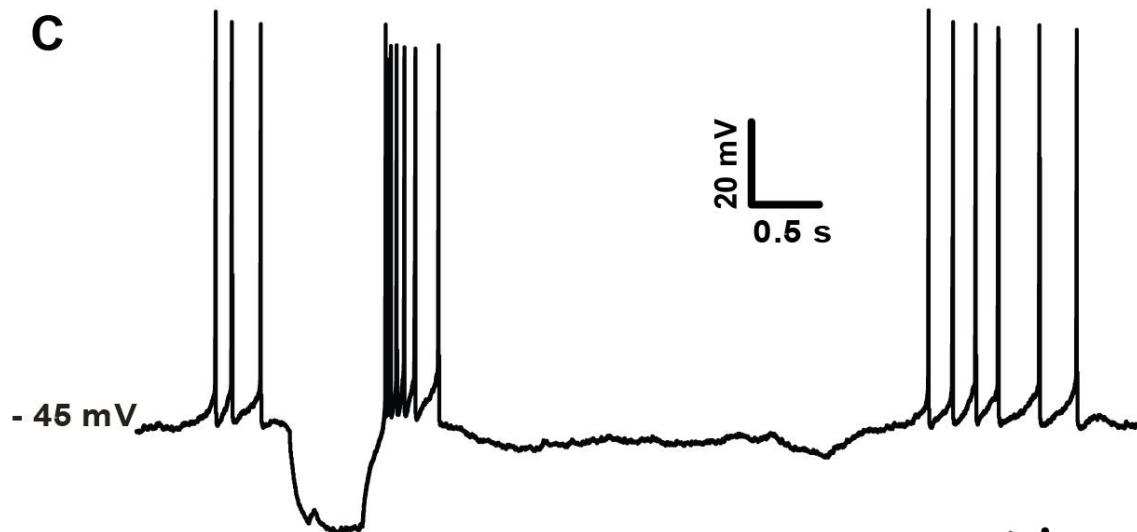
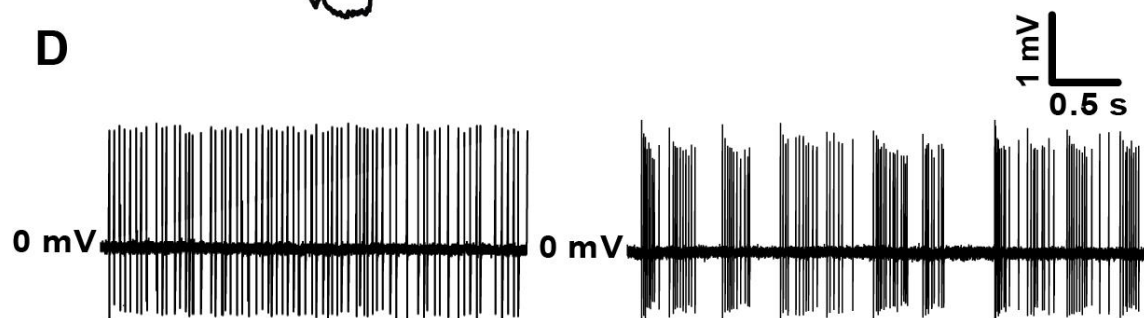
A**B****C****D**

Figure 3.1: Typical microscopic and electrophysiological properties of normal LTSI.

A. An image of a sagittal brain slice from NPY-GFP expressing BAC transgenic mice. In the striatum, GFP-positive neurons are either LTSIs or NGFIs. . Calibration bars: 200 μm (left panel), 50 μm (right panel) and 20 μm 567 (inset). B. Normal spontaneous firing of LTSI in a whole-cell recording (example for regular pattern). C. Low-threshold Ca^{2+} spikes in response to negative current injections. D. Biphasic spikes in LTSI during a cell-attached recording (regular and burst

3.3.2 Effects of blocking nicotinic and/or muscarinic receptors on LTSI activity:

First of all we were interested in investigating the effects of endogenously released acetylcholine in the striatum on the LTSI activity. Thus, selective nicotinic and/ or muscarinic receptor antagonists were applied to achieve this goal.

Atropine sulfate (20 μM) was used as a blocker for the muscarinic cholinergic receptors in the same concentration used in previous studies on striatal neurons (Pakhotin & Bracci, 2007). On the other hand, mecamylamine hydrochloride (10 μM) and nicotine (1 μM) were used to selectively block the nicotinic cholinergic receptors. Mecamylamine was used in the same concentration reported in other studies on striatal neurons (Salamone et al., 2014). Moreover, it has been shown that nicotine at low concentrations (1 μM) causes desensitization of the nicotinic receptors (Britt & McGehee, 2008). This desensitization of nicotinic receptors is similar to what occurs inside the body during nicotine addiction. However, nicotine preferentially desensitize non- $\alpha 7$ nicotinic receptors specially $\alpha 4\beta 2$ receptors (Giniatullin, Nistri, & Yakel, 2005).

In the first group of experiments, mecamylamine (10 μM) was applied to investigate the effects of blocking the nicotinic receptors on LTSIs. In 7/7 experiments, mecamylamine caused significant ($p < 0.05$) hyperpolarization of the LTSIs associated with significant ($p < 0.05$) decrease in their spontaneous firing (Figure 3.2A). The inter spike interval (ISI) for individual neurons was significantly ($p < 0.05$) increased (Figure 3.2D). The inhibitory effects of mecamylamine were reversed upon wash out of the drug ($n = 7$). In one cell-attached

recording, application of nicotine (1 μ M) caused an obvious decrease in the firing activity of the LTSl (Figure 3.2C).

Using a similar protocol, atropine (20 μ M) was applied to block the muscarinic receptors. In 6/9 experiments atropine led to a depolarization accompanied by a significant ($p<0.05$) increase in spontaneously firing frequency of LTSlS (Figure 3.2B). The ISI for individual neurons was significantly ($p<0.05$) decreased in these experiments (Figure 3.2D). The effects of atropine were reversed by washing out ($n = 6$). Application of 20 μ M atropine in one cell attached recording caused a marked increase in the firing activity of the LTSl that was reversed upon washout (Figure 3.2C).

From these data, it was concluded that the nicotinic effect of ACh on LTSlS is stimulatory. On the other hand, the muscarinic effect of ACh on LTSlS is inhibitory.

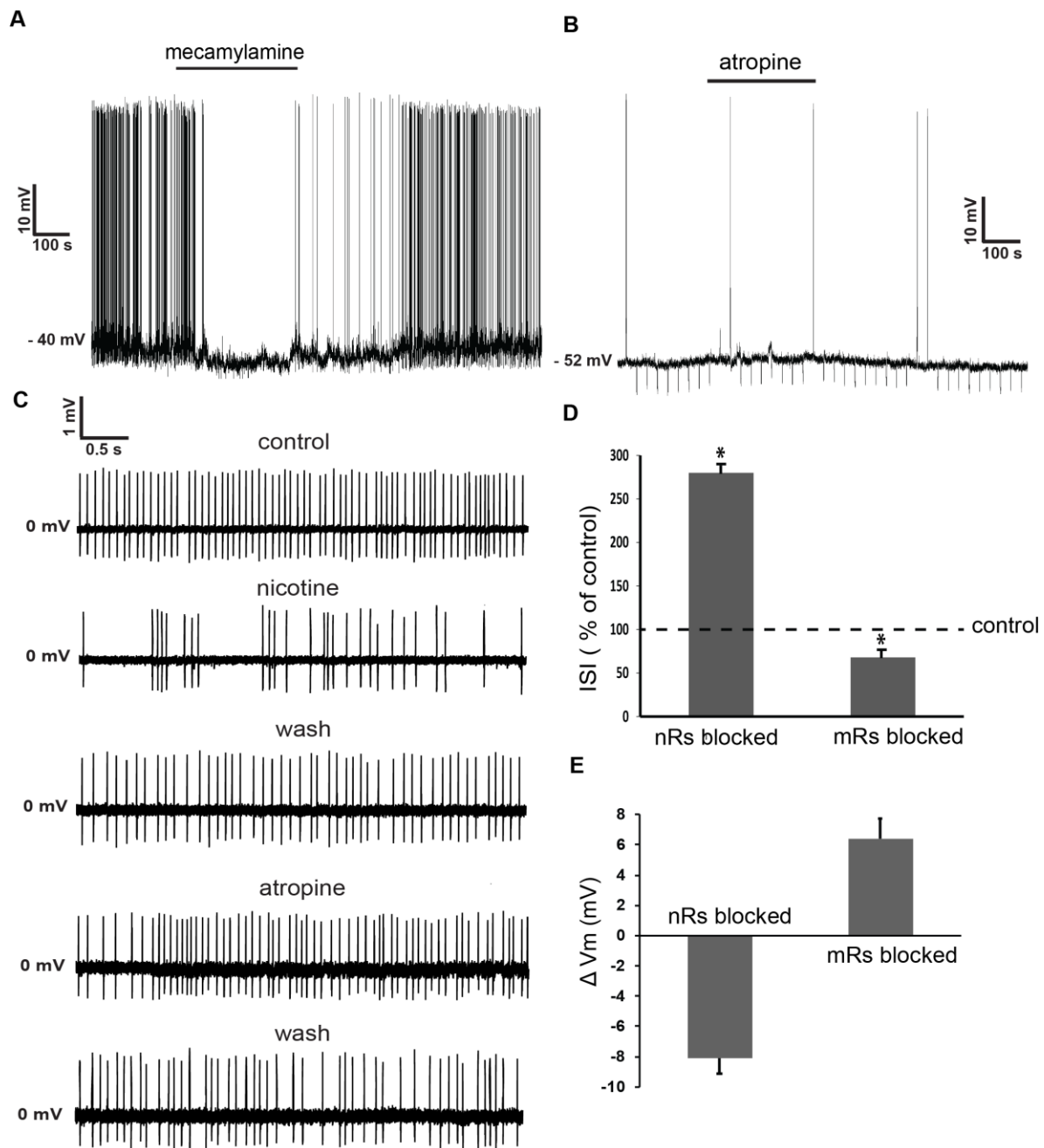


Figure 3.2: Effects of the nicotinic and muscarinic receptor blockers on LTSl activity.

A. Application of mecamlamine (20 μ M) caused reversible significant ($p < 0.05$) hyperpolarization of LTSl with significant decrease in the spontaneous firing. B. Applications of atropine (10 μ M) caused significant ($p < 0.05$) reversible depolarization of LTSl. The effect was reversed by washout. C. A cell- attached recording of LTSl showed a decrease of the spontaneous firing of LTSl with nicotine (1 μ M) and an increase of the spontaneous firing with atropine (10 μ M). These effects were reversed by washout. D. ISI increased significantly ($p < 0.05$) with nicotinic blockers ($n = 7$) and decreased significantly ($p < 0.05$) with muscarinic blockers ($n = 6$). E. Average change of membrane potentials with nicotinic receptor blockers and muscarinic receptor blocker.

Under physiological conditions acetylcholine act on both nicotinic and muscarinic receptors simultaneously. Thus the effects of blocking both the nicotinic and muscarinic receptors were tested. In 5/5 experiments mecamylamine and atropine caused hyperpolarization and significant ($p<0.05$) decrease in the firing frequency of the LTSIs (Figure 3.3A). Moreover, application of nicotine (1 μ M) and atropine (20 μ M) caused significant ($p<0.05$) decrease of the spontaneous firing of the LTSIs with hyperpolarization of their membrane potential in 7/11 LTSIs (Figure 3.3B).

It was concluded that blocking of both types of cholinergic receptors have a significant ($p<0.05$) inhibitory effects on LTSI activity in control solution. This suggests that, in control solution, the stimulatory effect of acetylcholine is more dominant than its inhibitory effect.

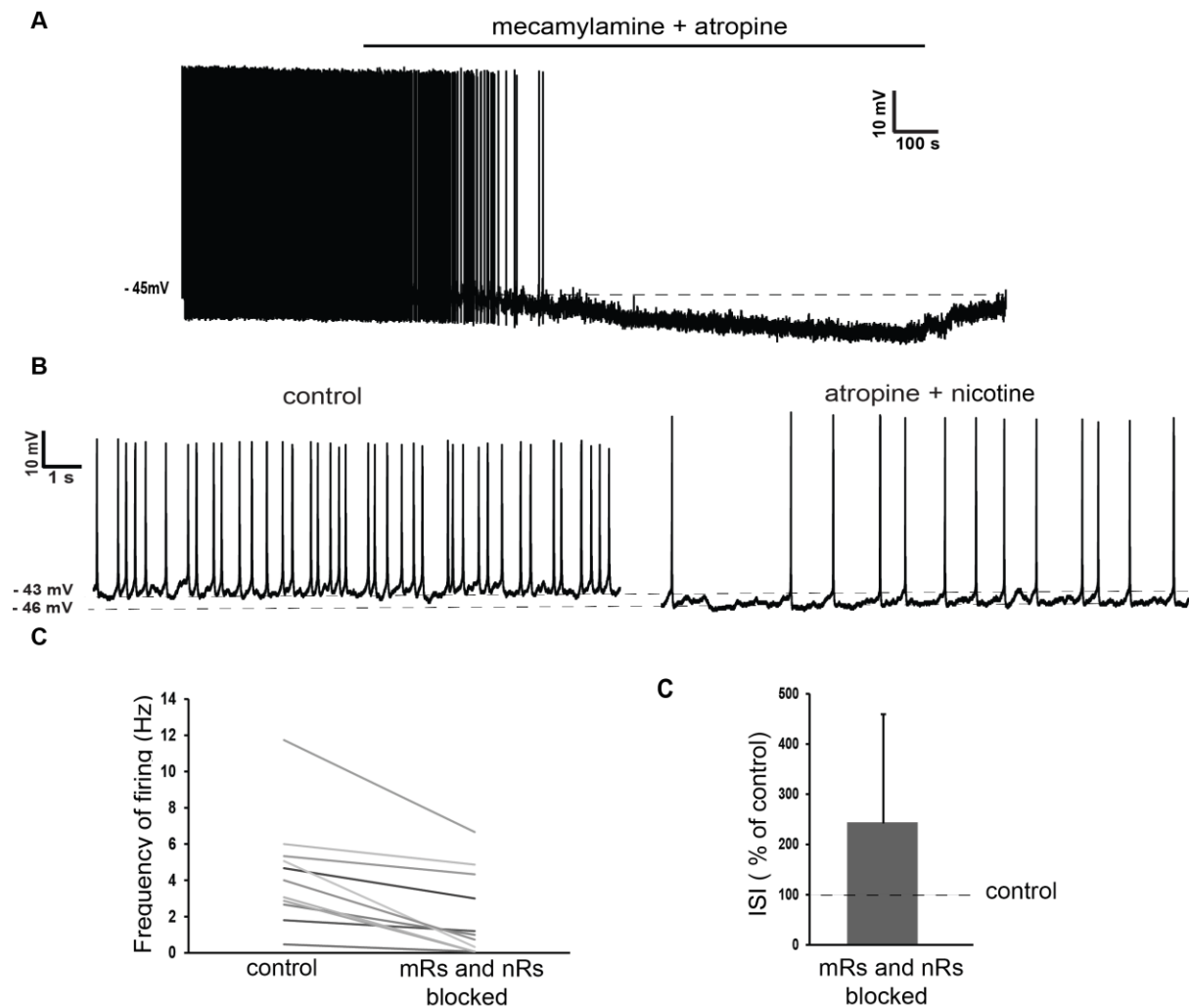


Figure 3.3: Simultaneous blocking of cholinergic nicotinic and muscarinic receptors inhibited LTSI activity.

A. Application mecamylamine and atropine inhibited a LTSI. B. Nicotine and atropine caused a hyperpolarization and significant ($p < 0.05$) decrease in LTSI spontaneous firing. C. Average change in the frequency of firing of LTSIs in control solution and after application of cholinergic receptor blockers in individual neurons. D. ISI significantly ($p < 0.05$) increased with cholinergic receptor blockers compared to normal.

3.3.3 Effects of blocking GABA_A receptors on LTSI activity:

It was reported that there is a tonic GABA release that modulate the MSN activity in the striatum. This tone is mediated through GABA_A receptors (Ade et al., 2008). As GABA is abundantly present in the striatum, it is expected that GABA tone can modulate the activity of other striatal neurons as well. Thus the effects of GABA on the LTSI activity were tested.

Therefore, GABA_A receptor blockers (picrotoxin and bicuculline) were used to block of the effects of tonic GABA release on LTSIs. Both drugs were used in the same concentrated reported previously in other studies on striatal neurons (Jiang & North, 1991).

In control solution, application of picrotoxin (100 μ M) resulted in significant ($p < 0.05$) reversible depolarization of the LTSIs with significant ($p < 0.05$) increase in the spontaneous activity in 5/5 experiments (Figure 3.4A). The effects of picrotoxin were reversed during the washout ($n = 5$). Similar results were obtained when bicuculline (25 μ M) was applied in 4/4 experiments.

TTX (1 μ M) was used in another group of experiments to block the action potential dependent release of neurotransmitters (Ade et al., 2008). It was observed that picrotoxin led to significant ($p < 0.05$) reversible depolarization of the LTSIs in 14/15 experiments (Figure 3.4B).

These experiments show that LTSIs are under tonic control of GABA. This tonic effect is mediated through GABA_A receptors. GABA is present in the extracellular environment even when spikes are blocked, presumably as a result of spike-independent vesicular release.

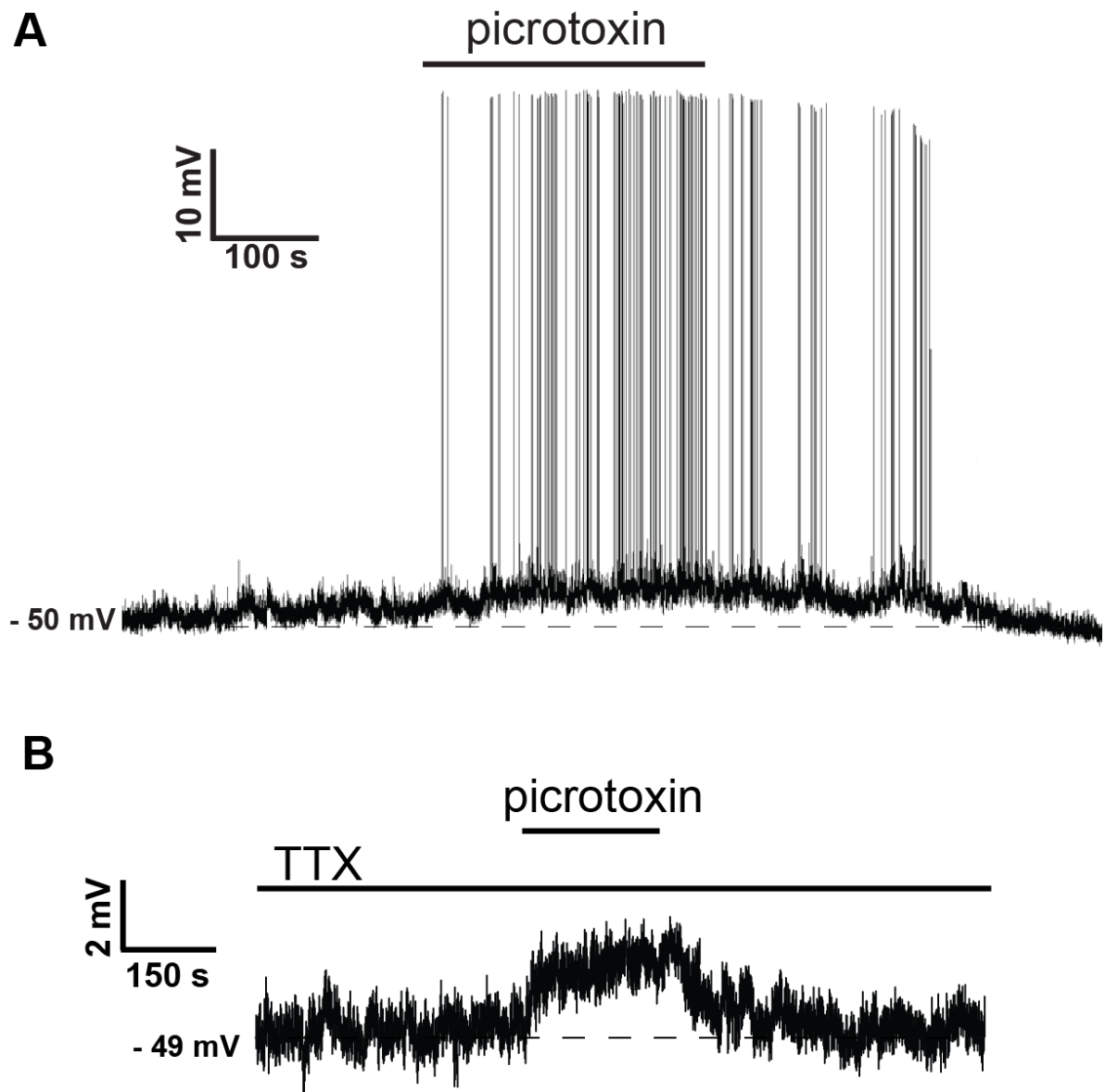


Figure 3.4: Blocking GABA_A receptors stimulates LTSIs.

A. Application of picrotoxin (100 μ M) in control solution caused significant ($p < 0.05$) reversible depolarization of LTSI with significant ($p < 0.05$) increase in the LTSI spontaneous activity. B. In presence of TTX, picrotoxin caused significant ($p < 0.05$) depolarization of another LTSI.

3.3.4 Direct effects of acetylcholine on LTSI activity:

The cholinergic modulation of LTSIs could be both direct and indirect. The direct effects include the action of acetylcholine on the muscarinic and nicotinic receptors on LTSIs cell membrane. On the other hand, the indirect effects may be mediated through other key modulators of the LTSI activity. In the first part of this study, the direct and indirect effects of acetylcholine on LTSIs were studied together in normal solution.

Accordingly, the next step was to investigate the direct effects of acetylcholine on LTSIs. This was possible by using TTX (1 μ M) and GABA_A receptor blockers to isolate the LTSIs from any synaptic effects.

It was found that application of mecamylamine (10 μ M) caused significant ($p < 0.05$) reversible hyperpolarization in 3/4 experiments (Figure 3.5A). Application of atropine (10 μ M) caused significant ($p < 0.05$) reversible depolarization in 4/4 experiments (Figure 3.5B). When both nicotinic and muscarinic receptor blockers were applied simultaneously significant ($p < 0.05$) depolarization in 3/4 LTSIs was observed (Figure 3.5C).

These experiments suggest that there is an endogenous cholinergic tone that modulates the LTSI activity all the time. In the presence of TTX, it is expected decreased levels of ACh that can also affect the balance between the muscarinic and nicotinic receptors. Thus, in the presence of TTX and GABA_A receptor blockers, the direct muscarinic effects become the more dominant than the nicotinic effects of acetylcholine.

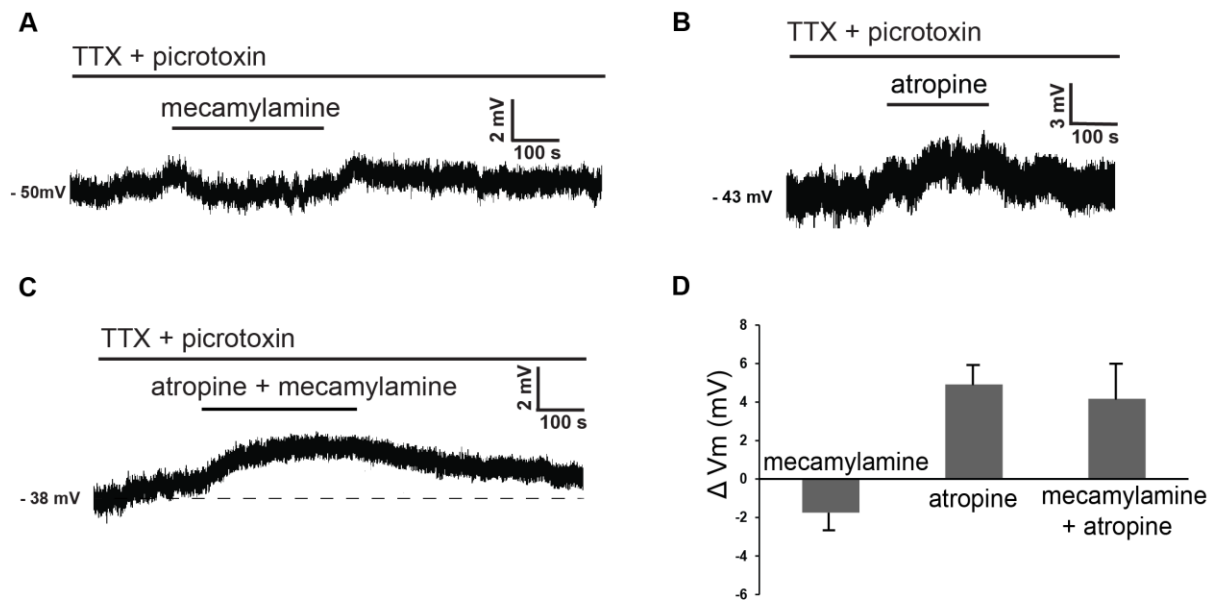


Figure 3.5: Effects of blocking nicotinic and/or muscarinic receptors on LTSI activity in presence of TTX and GABA_A receptor blockers.

A. Application of mecamylamine (20 μ M) in presence of TTX and picrotoxin caused significant ($p < 0.05$) reversible hyperpolarization of a LTSI. B. Application of atropine (10 μ M) in presence of TTX and picrotoxin caused significant ($p < 0.05$) reversible depolarization of another LTSI. C. Application of mecamylamine and atropine together caused significant ($p < 0.05$) depolarization of a LTSI. D. The average change in the membrane potential of LTSIs in the presence of mecamylamine ($n = 3$), atropine ($n = 4$) and both mecamylamine and atropine ($n = 3$).

3.3.5 Effects of cholinergic receptor blockers on GABAergic transmission on LTSIs :

From previous data, it was concluded that LTSI activity is affected by a tonic release of acetylcholine and GABA. The next step in our project was to study the effects of acetylcholine on the GABAergic neurotransmission on LTSI activity. This was done by testing the effects of cholinergic receptor blockers on spontaneous and evoked GABAergic IPSPs.

These experiments were carried out in the presence of glutamate receptor blockers D-AP5 (10 μ M; competitive NMDA receptor antagonist) and NBQX (10 μ M; selective AMPA receptor antagonist) to block the excitatory effects of glutamate on the LTSIs (John G Partridge et al., 2009).

In the first group of experiments, the effects of atropine (20 μ M) on the spontaneous and evoked GABAergic IPSPs were investigated. It was found that atropine significantly ($p < 0.05$) increased the spontaneous GABAergic events in 4/4 experiments (Figure 3.6A). There was a significant ($p < 0.05$) increase in the frequency of events; however, the amplitude of spontaneous events was not significantly changed (Figure 3.6B). Then the effects of atropine on the evoked GABAergic IPSPs were tested. Atropine significantly ($p < 0.05$) increased the evoked GABAergic IPSPs in 3/5 experiments (Figure 3.6C). Using Mann–Whitney U test, the paired pulse ratio before and after atropine was compared and a significant ($p < 0.05$) decrease in the paired pulse ratio was reported ($n=3$) (Figure 3.6C).

Using a similar protocol, the effects of mecamylamine (20 μ M) were tested. Mecamylamine caused significant ($p < 0.05$) decrease in the spontaneous activity in 3/3 experiments (Figure 3.6E). The frequency of the spontaneous events was significantly ($p < 0.05$) decreased. On the other hand, the amplitude of the spontaneous events was not significantly changed with mecamylamine (Figure 3.6D). Moreover, mecamylamine significantly ($p < 0.05$) decreased the evoked GABAergic IPSPs in 4/4 experiments (Figure 3.6F). However, there was no significant change in the paired pulse ratio with mecamylamine (Figure 3.6F).

These results suggest that ACh tends to facilitate the GABAergic transmission on LTSIs through the cholinergic nicotinic receptors. On the other hand, ACh tends to decrease the GABAergic transmission on LTSIs through the cholinergic muscarinic receptors.

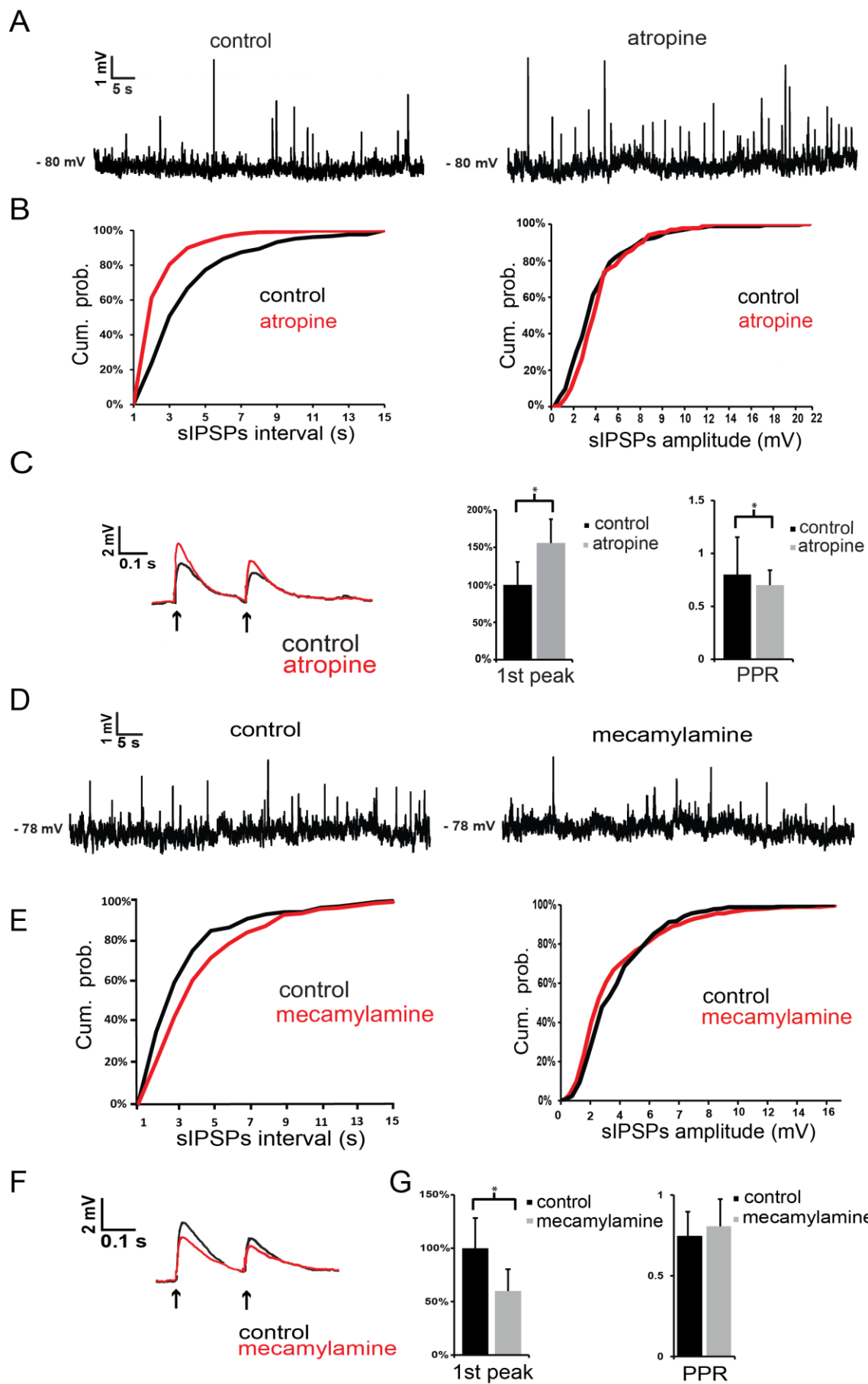


Figure 3.6: Effects of the cholinergic receptor blockers on GABAergic activity of LTSIs.

A. Atropine significantly ($p < 0.05$) increased the spontaneous GABAergic events on LTSI. B. atropine increased the frequency of spontaneous GABAergic events; however, there was no significant change in the average amplitude of the events. C. Atropine significantly ($p < 0.05$) increased the evoked GABAergic IPSPs. C. A diagram showing the average of normalized 1st peak amplitude before and after atropine. Also, the paired pulse ratio (PPR) was significantly ($p < 0.05$) decreased with atropine. D. Mecamylamine significantly ($p < 0.05$) decreased the spontaneous GABAergic events on LTSI. E. Mecamylamine significantly ($p < 0.05$) decreased the frequency of spontaneous GABAergic events ($p < 0.05$); however, there was no significant change in the average amplitude of the events. F. Mecamylamine significantly ($p < 0.05$) decreased the evoked GABAergic IPSPs. The average of normalized 1st peak before and after mecamylamine. Moreover, paired pulse ratio (PPR) was not significantly changed with mecamylamine.

In another group of experiments, the effects of simultaneous application of muscarinic and nicotinic receptor blockers on the GABAergic activity were examined. It was noted that application of atropine (20 μ M) and mecamylamine (10 μ M) caused significant ($p < 0.05$) increase in the spontaneous GABAergic activity in 3/4 cells (Figure 3.7A). Cholinergic receptor blockers significantly ($p < 0.05$) increased the frequency of spontaneous GABAergic activity but they did not significantly affect the amplitude of the events (Figure 3.7B). The cholinergic receptor blockers also significantly ($p < 0.05$) increased the evoked GABAergic IPSPs in 3/4 cells (Figure 3.7C). Using Mann–Whitney U test, the PPR was significantly ($p < 0.05$) increased with cholinergic receptor blockers ($n=3$) (Figure 3.7C).

These results show that the facilitatory effects of atropine on GABAergic transmission on LTSIs are larger than the inhibitory effects of mecamylamine. Thus, the net effects of cholinergic modulation of GABAergic transmission on LTSIs are inhibitory.

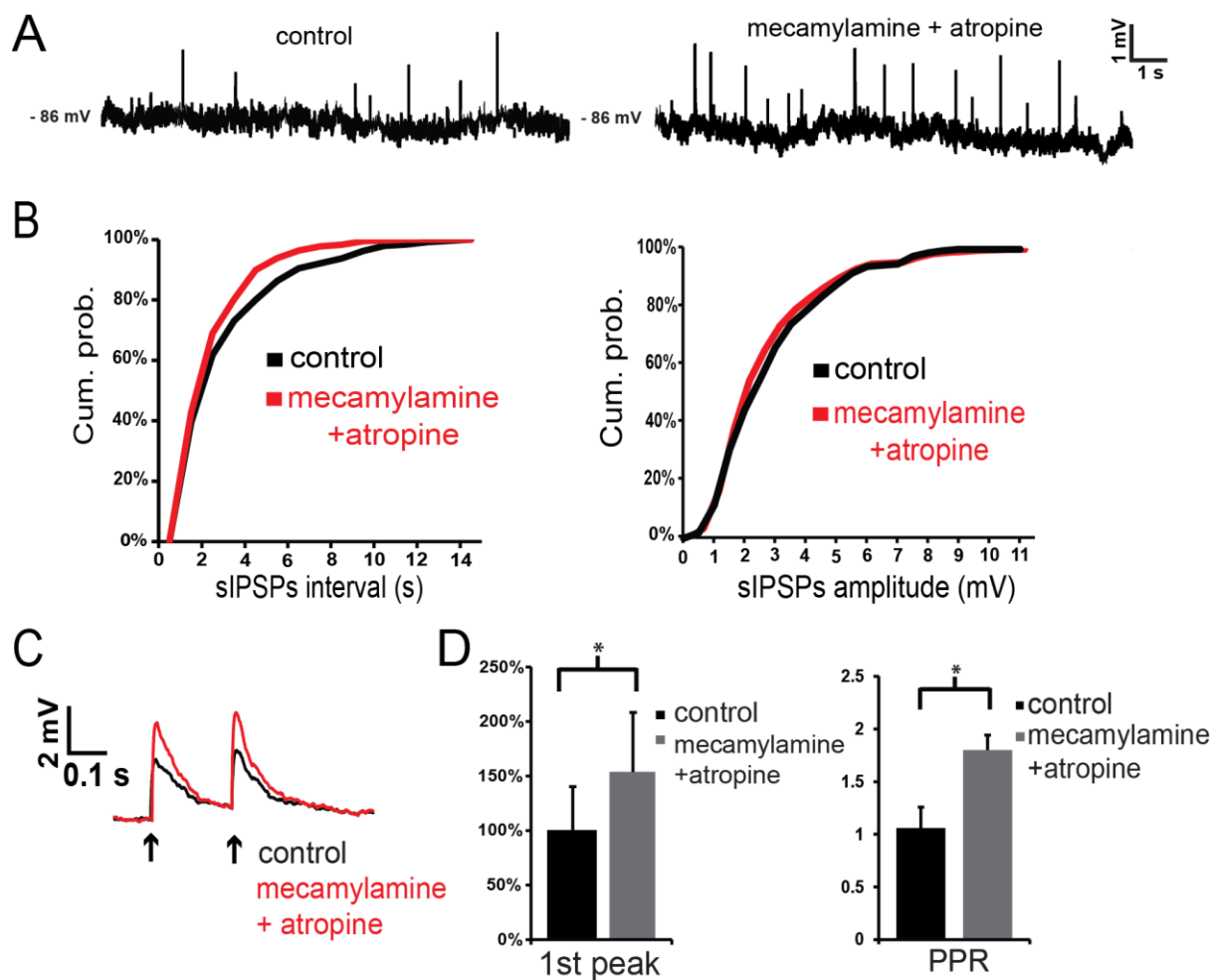


Figure 3.7: Blocking of cholinergic receptor increases spontaneous and evoked GABAergic events on LTSIs.

A. Atropine and mecamlamine together led to significant ($p < 0.05$) increased the spontaneous GABAergic events in a LTSI. B. Cholinergic blockers significantly ($p < 0.05$) increased the frequency of spontaneous events, however, cholinergic blockers did not affect the amplitude of the spontaneous GABAergic events. C. Atropine and mecamlamine significantly ($p < 0.05$) increased the evoked GABAergic IPSPs in another LTSI. E. Two diagrams; the first shows the average of normalized 1st peak before and after cholinergic blockers. The second one shows that PPR significantly increased with cholinergic blockers.

3.4 Discussion

The data of this study provide insight into the cholinergic control of LTSIs. This study demonstrates that LTSIs exhibit muscarinic and nicotinic responses. These responses are due to a complex interplay of direct effects of acetylcholine (acting on both muscarinic and nicotinic receptors) on LTSIs and indirect effects of the same receptors on GABA release.

Although cholinergic interneurons represent the main source of acetylcholine in the striatum, acetylcholine also comes from the projection axons from the forebrain (Toshihiko Aosaki et al., 2010). Using the ACh antagonists should block the effect of ACh in the striatum regardless the source.

It was found that blocking of cholinergic nicotinic receptors had inhibitory effects on LTSIs in control solution and in the presence of both TTX and GABA_A receptor blockers. On the other hand, blocking of the cholinergic muscarinic receptors had stimulatory effects on LTSIs in control solution and in presence of TTX and GABA_A receptor blockers.

When the cholinergic nicotinic and muscarinic receptors were blocked simultaneously, we observed that LTSIs were inhibited. However, in the presence of TTX and GABA_A receptor blockers, LTSIs were activated after blocking of the cholinergic receptors.

To investigate the underlying mechanisms for this dual effect of acetylcholine, we investigated the role of another neurotransmitter in the striatum; GABA. We reported the presence of a tonic effect of GABA on LTSIs. Application of GABA_A receptor blockers in control solution and in TTX led to significant ($p < 0.05$) depolarization of the LTSIs.

Furthermore, we investigated the cholinergic modulation of the GABAergic transmission on LTSIs. It was reported previously that activation of cholinergic interneurons indirectly increases release of GABA from GABAergic interneurons (De Rover et al., 2002; English et al., 2012; Sullivan et al., 2008; Witten et al., 2010). Luo and his colleagues (2013) showed that this increase is mediated through the nicotinic receptors. Moreover, they suggested that NPY-NFG and TH⁺ interneurons are the main source of tonic GABA affecting the MSNs (Luo et al., 2013).

Through a series of experiments, we found that application of cholinergic nicotinic receptor blockers decreased the GABAergic spontaneous IPSPs frequency but not the amplitude. Moreover nicotinic blockers decreased the evoked IPSPs. This agrees with previous data by Luo and colleagues that reported an excitatory effects of nicotinic agonists on LTSIs

mediated by GABA_A receptors (Luo et al., 2013). On the other hand, application of the cholinergic muscarinic receptor blockers increased the frequency of the sIPSPs but not their amplitude. Also, muscarinic receptor blockers increased the evoked GABAergic IPSPs.

Application of muscarinic and nicotinic receptor blockers simultaneously increased the frequency of sIPSPs but not the amplitude of these events. Cholinergic receptor blockers also increased the evoked GABAergic IPSPs.

From these results, we can conclude that the main effects of acetylcholine on GABAergic neurotransmission on LTSIs is the inhibitory effects mediated through the muscarinic receptors.

Our data suggests that LTSIs are under the control of tonic release of GABA and acetylcholine. These tones inhibit LTSIs and persist even in the absence of action-potential release of these transmitters.

When the full network is functioning, the effects of ACh on LTSIs become more complex. ACh can potentially modify many players in the network through the nicotinic and muscarinic receptors. These players can modify the LTSIs activity directly and indirectly.

Thus under control conditions, acetylcholine exerts a direct inhibitory effect on LTSIs and indirect stimulatory effect on the same neurons through modulation of striatal network specially the GABAergic transmission. The net balance between the direct and indirect effects of ACh on LTSIs tends to be stimulatory. This stimulatory effect mediated mainly through inhibition of GABAergic transmission on LTSIs that will lead to disinhibition of LTSIs.

The following diagram demonstrates the direct and indirect effects of acetylcholine on LTSIs. Furthermore, the implications of the data presented in this chapter on the overall basal ganglia function and pathology will be fully explored in the final discussion chapter of this thesis.

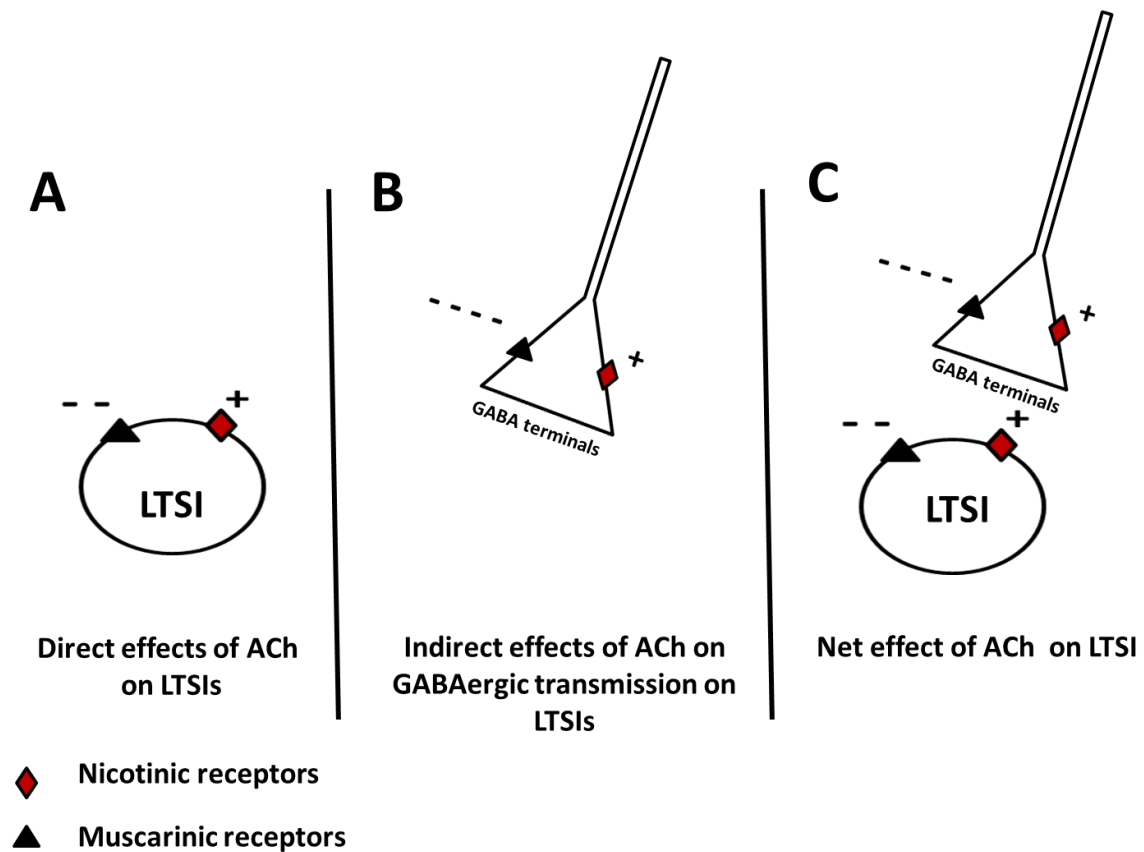


Figure 3.8: Direct and indirect effects of acetylcholine on LTSI activity.

- A. Direct effects of acetylcholine on LTSIs mediated through nicotinic and muscarinic cholinergic receptors.
- B. Indirect effects of acetylcholine on LTSIs are mainly mediated through modulation of the GABAergic transmission through nicotinic and muscarinic cholinergic receptors.
- C. The net effect of acetylcholine on LTSI activity occurs due to the balance between both direct and indirect effects of ACh.

Chapter Four: LTSI modulation of cholinergic interneurons activity.

4.1 Abstract

LTSIs are known to express the largest variety of neurotransmitters in the striatum. These transmitters include: GABA, NO, NPY and somatostatin. Each of these transmitters is reported to play an important role in modification of striatal function. On the other hand, we reported a strong cholinergic control of LTSI activity in-vitro. This study aims at studying how LTSIs can modulate the function of the cholinergic interneurons.

Using transgenic ChR2-EYFP-SOM-IRES-Cre mice, we were able to use blue light to selectively stimulate the LTSIs expressing somatostatin in the striatum. Whole-cell recordings of the cholinergic interneurons were carried out during the optogenetic stimulation of the LTSIs. It was observed that activation of LTSIs led to reversible depolarization of the cholinergic interneurons. This depolarization was not blocked by ODQ (10 μ M; selective inhibitor of NO-sensitive guanylyl cyclase), cyclosomatostatin (1 μ M; Non-selective somatostatin (sst) receptor antagonist), PD 160170 (20 μ M; selective neuropeptide Y1, Y2 and Y5 receptors ($K_i > 10$ μ M)). However, the depolarization was completely blocked by L-NAME (100 μ M; NO synthase inhibitor).

These results indicate that the LTSIs exert a main stimulatory effect on the cholinergic interneurons. This stimulatory effect is mediated by nitric oxide. The mechanism underlying the NO effect on cholinergic interneurons is through s-GMP independent mechanism.

The results of this study provide an insight into the interaction between LTSIs and cholinergic interneurons in the striatum.

4.2 Introduction

Optogenetics is the combination of genetics and optics that enable the control of specific events within a defined population of cells in the living tissue. Optogenetics represent a promising revolution that will enable us to study how neurons operate individually and as members of networks (Deisseroth, 2011).

Introducing light-responsive proteins into brain cells of living animals allowed neuroscientists to control neurons activity by turning them on or off with unprecedented precision. This approach also provided the opportunity to investigate the structure and function of neural networks. Optogenetics began with the discovery of the light activated cation channel channel-rhodopsin2 and the light activated chloride pump Halorhodopsin (NpHR) (Zhang et al., 2007).

When ChR2 is activated with blue light (λ_{max} 470nm), it acts as an inwardly rectifying cation channel causing depolarization of the cells. On the other hand, activation of NpHR by yellow light (λ_{max} 580nm) acts as hyperpolarizing Cl-pump that lead to hyperpolarization of the cells (Figure 4.1). These two light-responsive proteins form an ideal pair for the activation and inactivation of different neurons. Using different promoters and molecular biology methods, ChR2 and NpHR can be selectively expressed in certain neurons in the brain (Fenno, Yizhar, & Deisseroth, 2011).

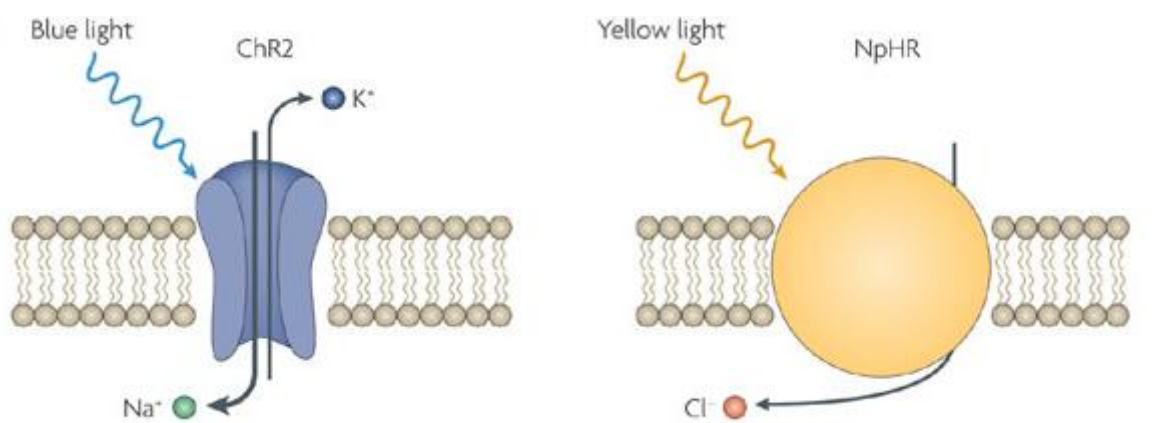


Figure 4.1 : Schematic diagram of the action of ChR2 and NphR on a neuron.

Blue light opens the ChR2, increasing intracellular cations (mostly Na^+ and low levels of Ca^{+2}) turning the neuron 'on'. On the other hand, yellow light introduces chloride ions into the cells leading to turning the neuron 'off'. Adapted from (Zhang, Aravanis, Adamantidis, de Lecea, & Deisseroth, 2007).

In this study we used transgenic ChR2-EYFP-SOM-IRES-Cre mice. In these mice, ChR2 was expressed in SOM expressing neurons in the striatum. As LTSIs are the only known source of somatostatin in the striatum (Muñoz-Manchado et al., 2014) , application of blue light allowed selective stimulation the LTSIs.

In the previous part of the research project we studied the cholinergic modulation of LTSIs. To complete the picture about the mutual interaction between the two TANS in the striatum (LTSIs and cholinergic interneurons), we decided to study the effects of optical activation of LTSIs on cholinergic interneurons.

LTSIs are known to express a wide variety of neurotransmitters that include: GABA, NO, NPY and somatostatin. However, little is known about the effects of these transmitters on the cholinergic interneurons.

A previous study suggests that LTSIs may be responsible for the recurrent inhibition in the cholinergic interneurons (Sullivan et al., 2008). In vivo microdialysis study reported that NO is involved in the control of release of ACh in the striatum (Guevara-Guzman et al., 1994) but the data related to this issue seems to be conflicting. Although, some studies reported that NO donors increased the striatal ACh release (Guevara-Guzman et al., 1994; H Prast et al., 1995; Helmut Prast & Philippu, 2001; H Prast et al., 1998), which was mediated partially by enhancing glutamate release. Many NO donors could not trigger the release of ACh in the striatum in the presence of glutamate receptors antagonists (H Prast et al., 1998; Sandor, Brassai, Pliskas, & Lendvai, 1995). Moreover, other studies reported that that endogenous NO decreased NMDA-induced ACh release (Ikemoto, 2007). However, it was reported that NO donors are capable of depolarizing the cholinergic interneurons through a c-GMP mechanism (Centonze et al., 2001).

This study aims at using the optogenetic stimulation of LTSIs. Understanding the mutual interaction between cholinergic interneurons and LTS interneurons will help in understanding more about the dynamics of the striatal network and how these spontaneously active neurons tunes the striatal output and basal ganglia functioning.

4.3 Results

4.3.1 Confirmation of ChR2 expression and experimental configuration :

The main aim of this study was to investigate LTSI modulation of cholinergic interneurons. Previously the effects of NO on cholinergic interneurons have been studied. However, this was done using NO donors (Centonze et al., 2001).

In our study, instead of NO donors, we tried to elicit NO release by stimulating LTSIs with light. Optical stimulation represents another alternative to the pharmacological activation/inhibition.

Examining the slices under the epifluorescence microscope revealed the presence of EYFP-positive cells in the cortex and to a lesser extent in the striatum. EYFP positive cells in the striatum are expected to be only LTSIs (Galarraga et al., 2007). The EYFP was more localized in the cell membrane of the cells than the cell cytoplasm (Figure 4.2A)

To confirm the selective expression of ChR2 on SOM positive neurons we recorded from EYFP positive cells and EYFP negative cells.

Different neuronal types were identified by their distinctive electrophysiological properties. We observed the following:

1. All EYFP- positive cells had the typical electrophysiological properties of LTSIs.
2. Some of the EYFP- negative cells showed the electrophysiological properties of LTSIs. This agrees with the fact that ChR2 was expressed by SOM positive cells and it is known that 85% of the LTSIs express somatostatin. However, LTSIs are the only striatal neurons that express somatostatin (Muñoz-Manchado et al., 2014).
3. Light stimulation of EYFP-positive cells exhibited light-evoked depolarization associated with generation of action potential when depolarization reached the threshold (Figure 4.2B). A train of blue light pulses of different durations was applied to produce action potentials. With light- activation more than 10 s, the cells usually enter in a depolarization block.
4. The EYFP- negative cells did not respond to light included MSNs, FSIs, cholinergic interneurons and NGF interneurons (Figure 4.2C).

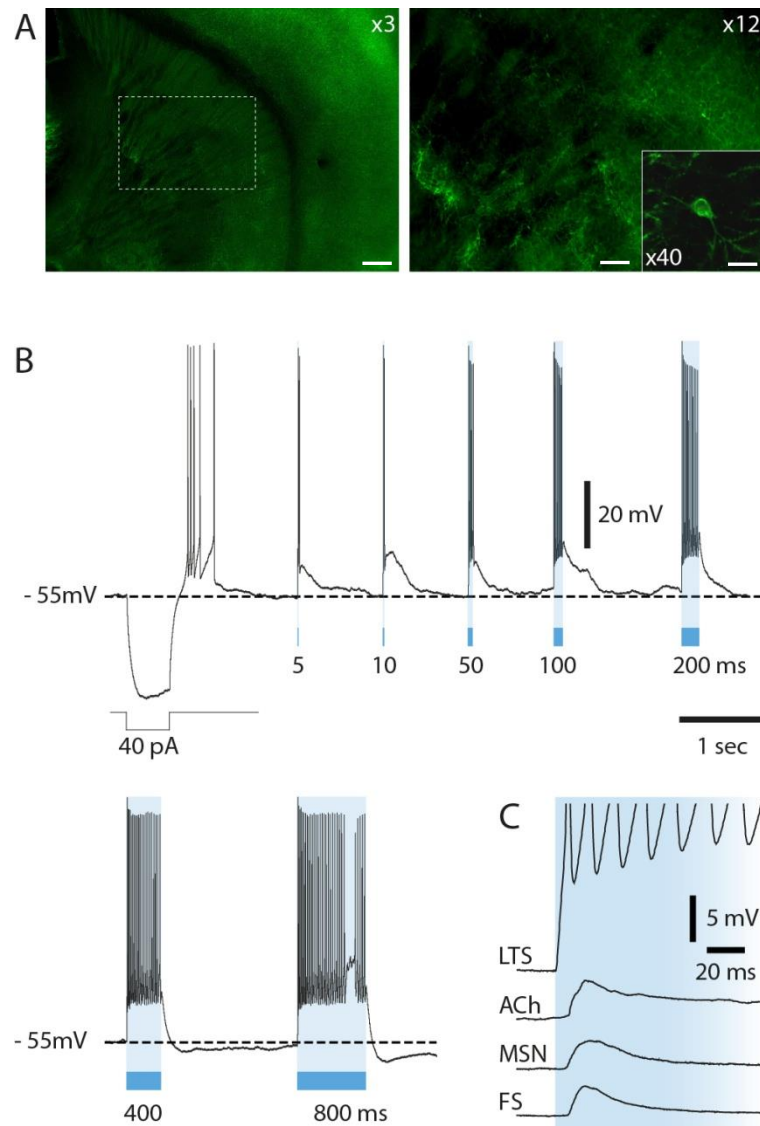


Figure 4.2: striatal neurons response to optogenetic stimulation.

A. Sagittal slices from ChR2-EYFP-SOM-IRES-Cre mouse shows that the fluorescence was localized in the SOM cells in the cortex and striatum. Calibration bars: 660 150 μm (left panel), 60 μm (right panel) and 25 μm (inset) B. The response of EYFP- positive LTSIs to optical stimulation. C. Response of EYFP- negative striatal neurons to optical stimulation.

4.3.2 Electrophysiological properties of cholinergic interneurons.

A 10 s blue- light stimulation of LTSIs was used to elicit the release of different transmitters from LTSIs. On the other hand, the effects of blue-light stimulation on cholinergic interneurons were examined. The light stimulation was repeated every 3 minutes to avoid desensitization of the NO receptors (Bellamy, 2000).

A total 11 ChR2-EYFP-SOM-IRES-Cre mice (both males and females) aged 28 ± 6 days were used in these experiments. Current-clamp recordings were obtained from 14 striatal cholinergic interneurons. We had 11 whole-cells, 3 cell-attached recordings.

During whole cell recordings, cholinergic interneurons were identified by their very large size relative to the other striatal neurons. This identification of cholinergic interneurons was confirmed after patching by their distinctive electrophysiological properties that include (Bennett et al., 2000) (Figure 4.3):

- i. Spontaneous firing activity without any stimulation.
- ii. Average membrane potential in absence of any injected current was -62 ± 2.7 mV.
- iii. Presence of after hyperpolarization (AHP) following a burst of spikes.
- iv. Input resistance ranged around $143 \pm 41\text{M}\Omega$.

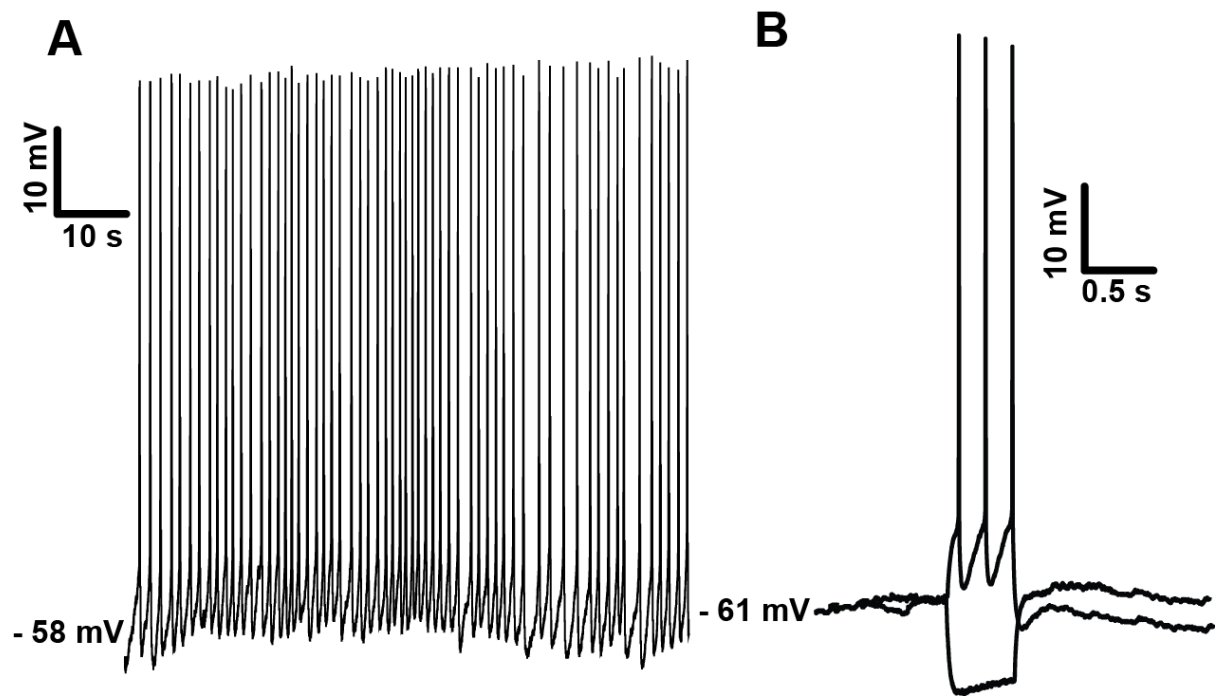


Figure 4.3: Electrophysiological properties of cholinergic interneurons.

A. Cholinergic interneurons electrophysiological properties include spontaneous firing. B. Electrophysiological properties of cholinergic interneurons; hyperpolarized membrane potential, after hyperpolarization and the depolarization sag.

4.3.3 Effects of optogenetic stimulation of LTSIs on cholinergic interneurons:

Application of blue light for 10 seconds with 3 minutes intervals caused robust depolarisations of the cholinergic interneurons with a marked increase in their firing frequency. The response of the cholinergic interneurons to photo-stimulation of LTSIs developed in average 27.3 ± 15.2 ms after the blue light was turned on. Moreover, the light-induced activation decayed 250 – 2500 ms after termination of photo-stimulation.

LTSIs are known to express several neurotransmitters that could potentially be responsible for the depolarization of cholinergic interneurons, including somatostatin, NPY and NO (Beatty et al., 2012).

In order to test the involvement of NO, we first tested the involvement of NO through sGC pathway. We used ODQ that was reported to block the depolarization of cholinergic interneurons induced by NO donors (Centonze et al., 2001). However, in our experiments, ODQ failed to affect light-induced depolarization in 3/3 cholinergic interneurons.

Adding cyclosomatostatin (1 μ M; Non-selective somatostatin receptor antagonist), PD 160170 (20 μ M; neuropeptide Y1, Y2 and Y5 receptors blocker) and ODQ (10 μ M; selective GC inhibitor) together, did not block the light-induced depolarization of the cholinergic interneurons in 4/4 experiments (Figure 4.4).

These results show that LTSIs exert a stimulatory effect on cholinergic interneurons. This stimulatory effect is not mediated through somatostatin or NPY. Moreover, the stimulatory effect of LTSIs on cholinergic interneurons not mediated through NO acting through sGC dependent mechanism.

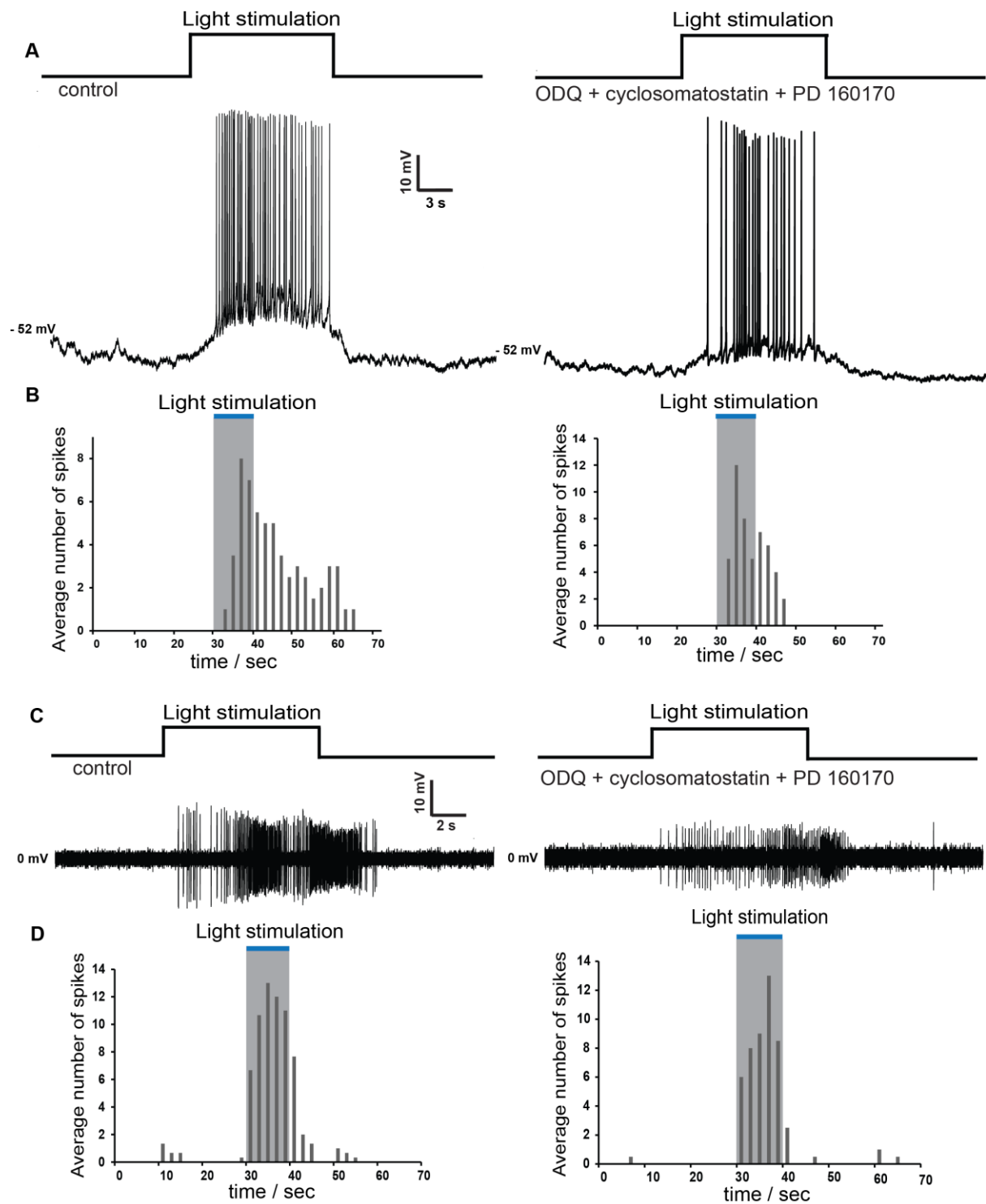


Figure 4.4: Optogenetic stimulation of LTSIs stimulated the cholinergic interneurons.

A. Whole cell recording from a cholinergic interneurons shows that light stimulation of slices from Chr2-EYFP-SOM-IRES-Cre mouse caused marked reversible depolarization with marked increase in the firing rate of the cholinergic interneurons. The light-induced depolarization was not blocked by ODQ, cyclosomatostatin or PD160170. B. diagrams that represent the average number of spikes in 3 cycles before, during and after light stimulation in control solution (left) and in presence of ODQ, cyclosomatostatin and PD 160170. C. A cell attached recording from a striatal cholinergic interneuron shows the effect of blue light stimulation in control solution (left) and in presence of ODQ, cyclosomatostatin and PD 160170. D. diagrams that represent the average number of spikes in 3 cycles of photo-stimulation before, during and after light stimulation in control solution (left) and in presence of ODQ, cyclosomatostatin and PD 160170.

In another group of experiments, the NO synthase inhibitor L-NAME hydrochloride (100 μ M) was applied in the same concentration used in other studies in the striatum (Hartung, Threlfell, & Cragg, 2011). L-NAME completely blocked the light-induced depolarization of the cholinergic interneurons in 4/4 experiments (Figure 4.5 A and C).

These results suggest that NO mediate the stimulatory effects of LTSIs on cholinergic interneurons through a sGC independent mechanism.

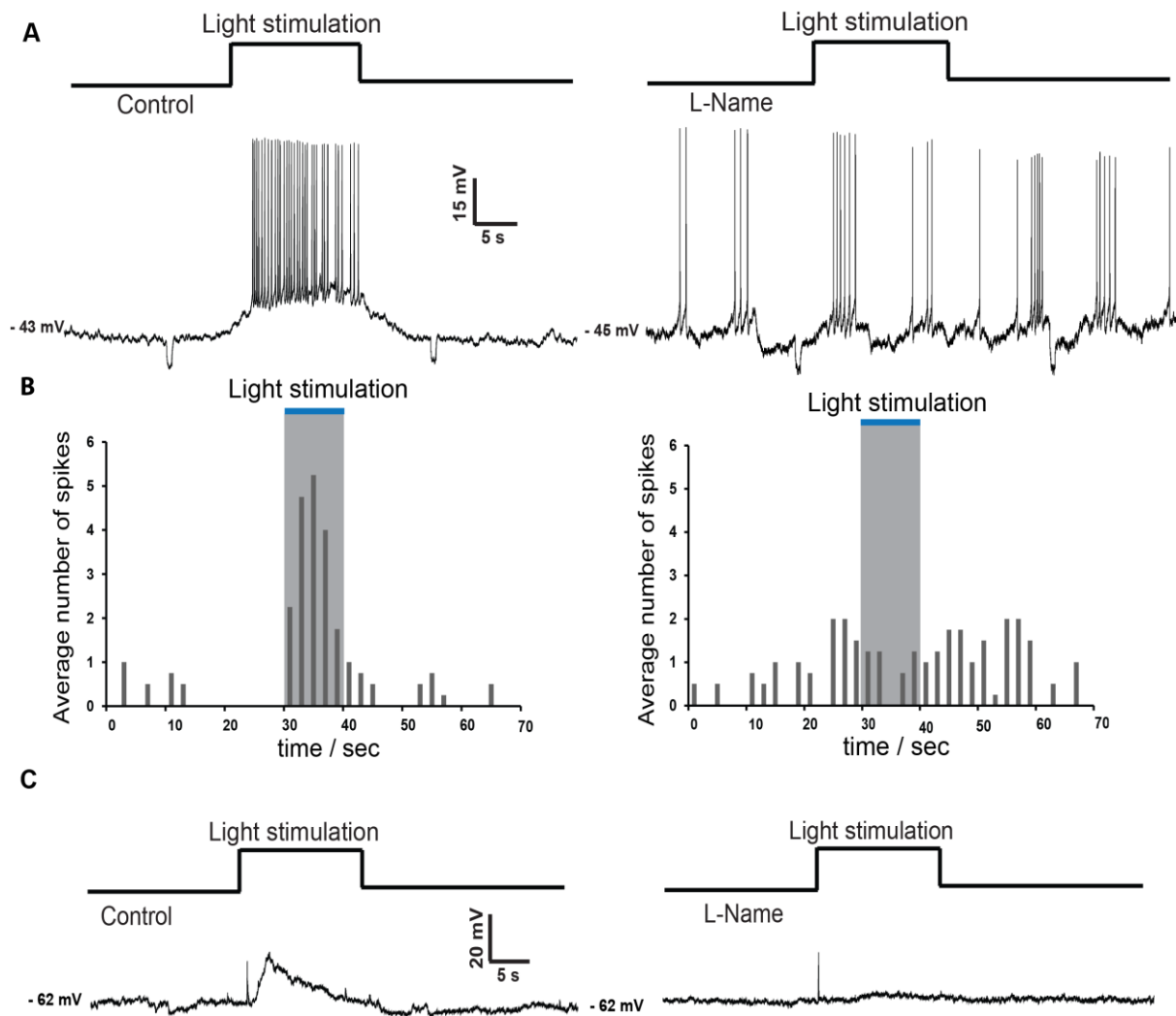


Figure 4.5: L-Name blocks the light-induced depolarization of cholinergic interneurons

A. A whole cell recording from a cholinergic interneuron shows that L-NAME completely blocked the stimulatory effect of LTSIs stimulation. B. A diagram shows the average number of spikes before, during and after blue light stimulation under control conditions (left) or in presence of L-NAME (3 cycles in each condition). C. Another example of whole cell recording of cholinergic interneurons in control solution (left) and with L-NAME (3 cycles in each case).

4.4 Discussion

The data of this study provide insight into the LTSI modulation of the cholinergic interneurons. Our data indicate that LTSI activation has strong excitatory effects on cholinergic interneurons. This light-induced excitation is mediated by NO released from the activated LTSIs. Moreover, our results show that the effects of nitric oxide do not depend on activation of GC pathways.

Using optogenetics, blue light was used to specifically activate the LTSIs expressing somatostatin. This activation of LTSIs is expected to be associated with the release of many neurotransmitters expressed by these interneurons.

In our study light-induced depolarization were not blocked by ODQ (selective inhibitor of NO-sensitive guanylyl cyclase), cyclosomatostatin (Non-selective somatostatin receptor antagonist) or PD 160170 (selective neuropeptide Y1, Y2 and Y5 receptors blocker). However, the depolarization was completely blocked by L-NAME hydrochloride. These results demonstrate that endogenous NO released from LTSIs acts to stimulate the cholinergic interneurons through CG-independent mechanisms. This is consistent with results reported from Hartung and colleagues who reported an increase in the dopamine release in the nucleus accumbens with NO donors. The stimulatory effects of NO donors were not blocked by ODQ. NO effects were mediated through CG-independent mechanisms (Hartung et al., 2011).

On the other hand, Centonze and colleagues reported an activation of the cholinergic interneurons by NO donors (Centonze et al., 2001). They provide evidence that the stimulatory effects of NO donors on cholinergic interneurons are mediated through a CG-dependent mechanisms that was completely blocked by ODQ (Centonze et al., 2001). However, this study used a pharmacological approach through application of NO donors in to the brain slices.

Blue-light activation of LTSIs is expected to cause release of many transmitters released by these neurons. These transmitters act through different families of receptors to modify the cholinergic interneurons activity directly and indirectly. However, in our study the main reported effect of the light activation of LTSIs seems to be through NO release. These results cannot exclude the role of other transmitters released by LTSIs. More experiments

need to be done to reach a final conclusion about the role of other neurotransmitters released by LTSIs.

This information deepens our understanding about the mutual control of LTSIs and cholinergic interneurons. Moreover, the implications of these results on the overall basal ganglia function and pathology will be fully explored in the final discussion chapter of this thesis.

Chapter Five: opioidergic control of cholinergic and low threshold spiking interneurons in the striatum

5.1 Abstract

Opioid receptors and their ligands enkephalin and dynorphin play an important role in the striatum. Opioids are involved in the processing of reward and drug addiction. Opioids are also expected to play an important role in modifying the final striatal output. This study aimed at studying the opioidergic control of cholinergic interneurons and LTSIs. Both interneurons represent the TANs in the striatum. TANs are proved to play an essential role in reward processing.

Using transgenic mice in which NPY-expressing neurons are marked with green fluorescent protein (GFP), we investigated the effects of DPDPE (a δ receptor agonist) and (-)-U-50488 hydrochloride (a κ receptor agonist) on cholinergic interneurons. We also studied the effects of DAMGO (a μ receptor agonist), DPDPE (a δ receptor agonist) and (-)-U-50488 hydrochloride (a κ receptor agonist) on LTSIs activity.

DPDPE (1 μ M) and (-)-U-50488 hydrochloride (10 μ M) caused significant ($p < 0.05$) decreased in the spontaneous firing of cholinergic interneurons.

On the other hand, DAMGO (1 μ M) produced hyperpolarizing effects in 73% of LTSIs and depolarizing effects in 6% of LTSIs tested when applied in control solution. We investigated the cause of this DAMGO-induced depolarization in a subpopulation of LTSIs. The dual effects of DAMGO persisted in the presence of tetrodotoxin (TTX), a sodium channel blocker. However, when GABA_A, nicotinic & muscarinic receptors were additionally blocked, DAMGO effects on LTSIs were always hyperpolarizing. It was also found that DAMGO decreased the GABAergic transmission on LTSIs. These findings suggest that μ -receptor agonists affect LTSIs both directly (exerting inhibitory effects) and indirectly through inhibition of GABA and acetylcholine release.

Moreover, DPDPE (1 μ M) significantly ($p < 0.05$) hyperpolarized 100% of tested LTSIs tested in control solution and in TTX. Also, DPDPE (1 μ M) decreased the GABAergic transmission on LTSIs. Furthermore, (-)-U-50488 hydrochloride (20 μ M) inhibited 100% of tested LTSIs in control and TTX solution. However, (-)-U-50488 hydrochloride did not cause any significant change in the GABAergic transmission on LTSIs.

These results provide insight into the effects of different opioid peptides on striatal microcircuits through modifying the LTSIs, cholinergic interneurons and GABAergic transmission.

5.2 Introduction

The striatum consists of two distinct neurochemical compartments : patch and matrix (Lovinger, 2010). Striatal patches are characterized by rich μ -opioid receptor binding, substance P staining, and poor staining for cholinergic markers. On the other hand, The matrix is characterized by dense acetylcholinesterase and choline acetyltransferase staining (A. Graybiel, Baughman, & Eckenstein, 1986), and immunoreactivity for calbindin and somatostatin (Gerfen, 1992). Striatal interneurons represent about 5% of total striatal neurons. However, these interneurons play an important role in modifying the final striatal output. Out of these interneurons cholinergic interneurons and LTSIs are in the focus of this study.

On the other hand, opioids are present in the striatum. It was found that dynorphin is expressed by the striatonigral MSNs, while enkephalin was expressed by the striatopallidal MSNs (Gerfen, 1992; Gertler et al., 2008). The locally released opioids have been in the focus of many studies (C. P. Blomeley & Bracci, 2011; Z. Jiang & R. North, 1992; Ma et al., 2012).

Jiang & North (1992) reported that μ and δ opioid receptors agonists presynaptically inhibit glutamatergic afferents to MSNs. Moreover, they reported that the presence of a subpopulation of striatal neurons, most probably interneurons, that are inhibited by a δ receptor agonist, whereas μ receptor agonists were ineffective (Jiang & North 1992). Furthermore, it was reported that postsynaptic opioid receptor activation, most probably μ receptors, inhibited MSNs through inhibition of the high-voltage activated calcium currents (Stefani, Surmeier, & Bernardi, 1994).

Moreover, studies suggested that activation of μ receptor leads to inhibition of glutamatergic innervations in patch and matrix compartments, while μ receptor inhibition of GABAergic terminals was observed in the patches only (Miura et al., 2007). Moreover, paired recordings from striatal MSNs showed that endogenously released opioids decrease the inputs from cortex, through presynaptic activation of μ receptors (C. P. Blomeley & Bracci, 2011).

Moreover, a role of endogenously released enkephalin in regulation of cholinergic transmission, through μ receptor activation was reported (Lendvai, Sandor, & Sandor, 1992). Furthermore, anatomical studies showed that axonal terminals expressing enkephalin form symmetrical synapses on the soma and dendrites of cholinergic interneurons (Martone, Armstrong, Young, & Groves, 1992). Moreover, it was demonstrated that opioids decrease

the release of ACh in the striatum (Arenas, Alberch, Arroyos, & Marsal, 1990; Maritza Jabourian et al., 2005). Recently, it was shown that μ receptor agonists inhibited the cholinergic interneurons (Ponterio et al., 2013).

More information about the modulation of striatal microcircuit by opioids is still needed. Previous study reported the inhibitory effect of enkephalin on MSNs and cholinergic interneurons. Moreover, the modulation of μ agonists on glutamatergic inputs was studied. On the other hand, we do not have any information about the effects of opiate peptides on other striatal interneurons. Also, the effects of opioids on GABAergic transmission on striatal interneurons are unknown. Understanding the opioidergic modulation of striatal interneurons, especially LTSIs, will provide important information about effect of opiates on the final striatal output.

Using transgenic mice in which LTSIs can be identified through a GFP attached to the NPY promoter, we studied the effects of DPDPE (a δ receptor agonist) and (-)-U-50488 hydrochloride (a κ receptor agonist) on cholinergic interneuron activity. Moreover, the effects of DAMGO (a μ receptor agonist), DPDPE (a δ receptor agonist) and (-)-U-50488 hydrochloride (a κ receptor agonist) on LTSIs activity were investigated. We also studied the indirect effects of opioids on LTSIs mediated through modulation of the GABAergic transmission on LTSIs.

5.3 Results

A total 127 BAC-NPY mice (both males & females) aged 23 ± 8 days were used in these experiments. Current-clamp recordings were obtained from 141 striatal GFP-LTSIs neurons; 97 of these recordings are whole cell recordings, 28 perforated-patch recordings and 16 cell-attached recordings.

5.3.2.1 Effects of δ and κ opioid receptor agonists on the cholinergic interneurons activity :

In chapter 3 and 4 we described the mutual interaction between the cholinergic and LTSIs interneurons. Our results showed that cholinergic interneurons exert a strong modulatory effect on LTSIs that can be detected even in the presence of TTX. On the other hand, strong photo-stimulation of LTSIs was needed to detect the light-induced depolarization of the cholinergic interneurons.

In order to add to the picture of how TANs contribute to the striatal network, we decided to study the effects of opiate peptides on cholinergic interneurons before studying the effects of opiates on LTSIs. Since the effects of μ receptor agonists on cholinergic interneurons has been described before (Ponterio et al., 2013), we only studied the effect of δ and κ on cholinergic interneurons.

5.3.1.1 Effects of δ receptor agonist on cholinergic interneuron activity in control solution:

Blomely and Bracci (2005) reported that cholinergic interneurons exhibit a rapid rundown of their properties when recorded with whole-cell recording techniques (C. Blomeley & Bracci, 2005). Hence we used amphotericin perforated- patch technique to test the effects of opiates on cholinergic interneurons.

DPDPE (1 μ M) was used as a δ receptor agonist. This concentration is similar to that used in previous studies on the basal ganglia neurons (Ogura & Kita, 2000).

In control solution, it was found that 7/7 cells, DPDPE led to significant decrease of the spontaneous firing activity the cholinergic interneurons associated with hyperpolarization of

the membrane potential (Figure 5.1A). The effects of DPDPE were partially reversed by applying specific δ receptor antagonist (SDM25aN; 1 μ M) (n = 4) or by washout (n = 3). The recovery was more observed in the membrane potential changes. Average change in membrane potential with DPDPE was $-4.4 \text{ mV} \pm 2.8 \text{ mV}$. Average recovery with SDM25N was $2.3 \pm 1.4 \text{ mV}$ while average recovery by washout was $2.6 \pm 0.8 \text{ mV}$.

This data suggests that δ receptor agonists exert a powerful inhibitory effect on cholinergic interneurons.

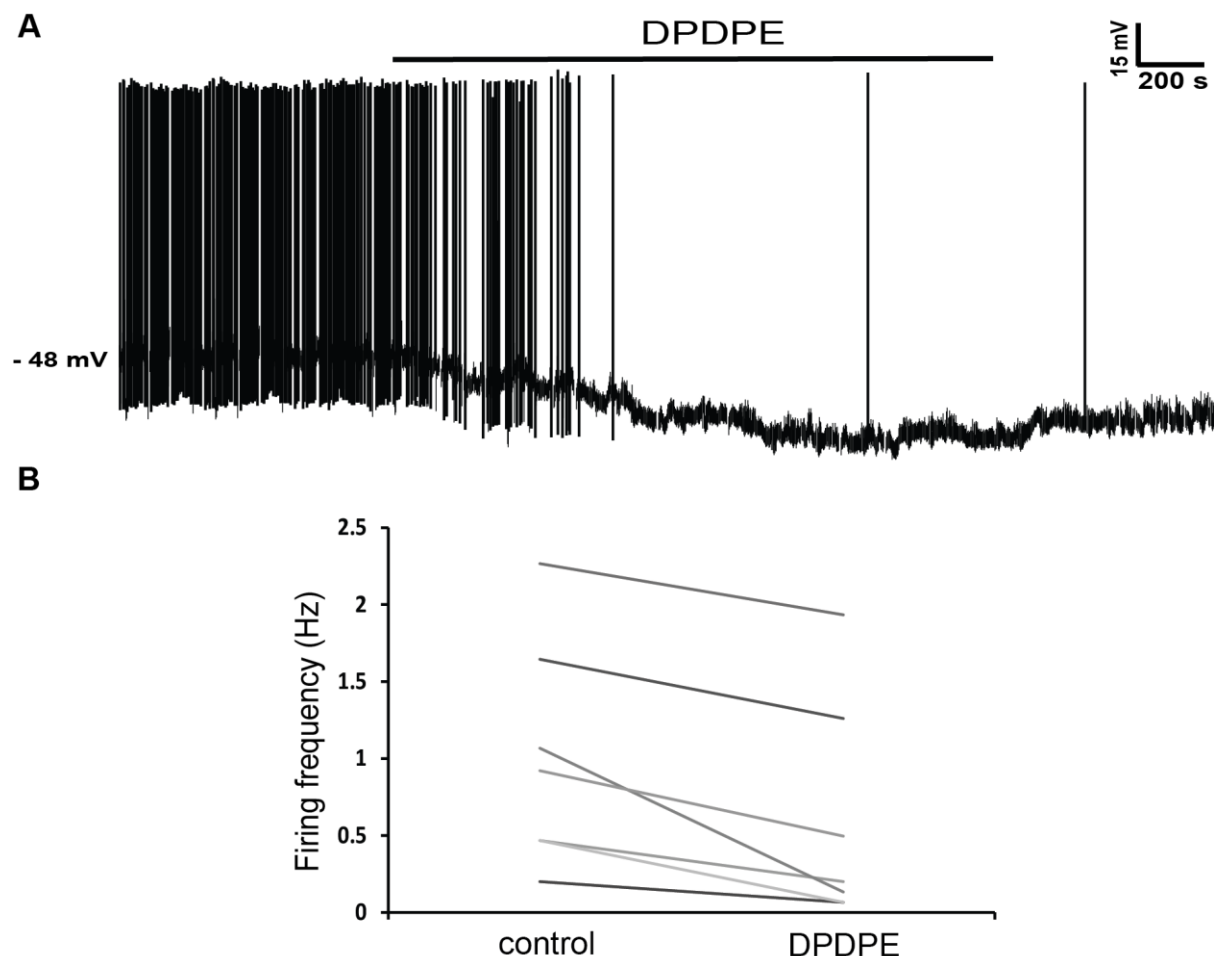


Figure 5.1: DPDPE (1 μ M) inhibits cholinergic interneurons.

A. DPDPE (1 μ M) significantly ($p < 0.05$) decreased cholinergic interneuron spontaneous activity and caused hyperpolarization in membrane potential. B. Effect of DPDPE on firing frequency in individual cholinergic interneurons (n = 7).

5.3.1.2 Effects of κ receptor agonist on cholinergic interneuron activity in control solution:

As a κ receptor agonist, U50488H (10 μ M) was used in the same concentration used in previous studies on in-vitro brain slices (Müller, Hallermann, & Swandulla, 1999). Using amphotericin perforated-patch technique, U50488H caused a significant ($p < 0.05$) decrease in the spontaneous firing activity associated with hyperpolarization of the membrane potential of cholinergic interneurons in 6/7 (Figure 5.2A). The effects of U50488H were partially reversed by applying specific κ receptor antagonist (GNTI dihydrochloride; 1 μ M) ($n = 3$) or by washout ($n = 3$). Moreover, average change in membrane potential with U50488H was -3.9 ± 2.2 mV. Similar to DPDPE, the recovery with U50488H was more observed in the changes in the membrane potential. The average of membrane potential recovery with GNTI dihydrochloride was 4.2 ± 1.2 mV, while the average of membrane potential recovery by washout was 3.8 ± 1.5 mV.

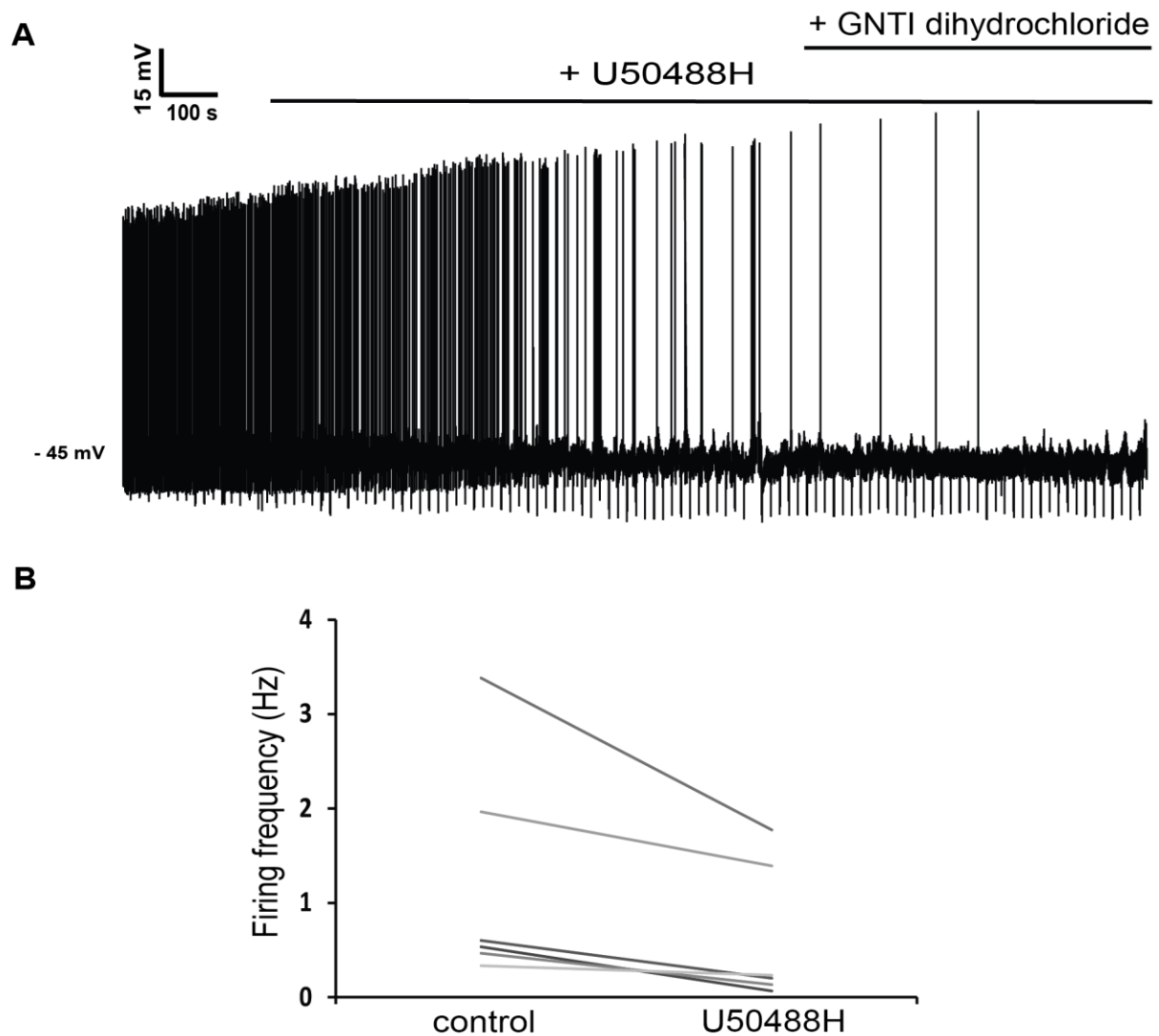


Figure 5.2: κ -receptor agonist (U500488H; 10 μ M) inhibits cholinergic interneurons.

A. U500488H (10 μ M) reversibly blocked cholinergic interneuron spontaneous activity and caused hyperpolarization in membrane potential (perforated- patch recording). B. Effect of U50488H on firing frequency in individual cholinergic interneurons (n = 6).

5.3.2 Effects of μ , δ and κ receptor agonists on LTSI activity:

After investigating the effects of δ and κ opioid receptor agonists on cholinergic interneurons, we studied the effects of μ , δ and κ agonists on LTSI activity.

5.3.2.1 Effects of μ receptor agonist (DAMGO) on LTSI activity :

5.3.2.1.1 Effects of μ receptor agonist on LTSI activity in control solution :

In the first group of experiments, 1 μ M DAMGO (μ receptor agonist) was applied in the same concentration used in previous studies on striatal neurons (Barral, Mendoza, Galarraga, & Bargas, 2003). In 8/12 whole-cell recordings, DAMGO caused a significant ($p < 0.05$) decrease in the firing frequency which caused complete cessation of spontaneous firing activity in the cells (Figure 5.3A). On the other hand, in 1/12 experiment, DAMGO caused a significant ($p < 0.05$) depolarization of the LTSI (Figure 5.3C). In the remaining 3/12 experiments, there were no significant change in spontaneous firing or the membrane potential. The effects of DAMGO peaked 11.5 ± 4.6 min after start of the application. The effects of DAMGO were partially reversed by wash ($n = 5$) or by applying the μ -receptor selective antagonist CTOP (1 μ M) ($n = 2$) or μ -receptor non selective antagonist naloxone (10 μ M) ($n = 2$). The average of firing frequency in control was 3.3 ± 2.3 . With DAMGO the average of firing frequency was 1.3 ± 0.9 . After recovery, the average of firing frequency was 2.6 ± 1.4 with washout, 1.7 ± 0.3 with CTOP and 1.9 ± 0.9 with naloxone.

To confirm these results, cell-attached configuration was used to minimize the interference with cell internal milieu caused by whole cell recordings. In 3/3 experiments it was found that DAMGO significantly ($p < 0.05$) decreased the firing frequency. A representative example of DAMGO effect is shown in (Figure 5.3).

These results show that DAMGO exerts dual effects on LTSIs in control solution.

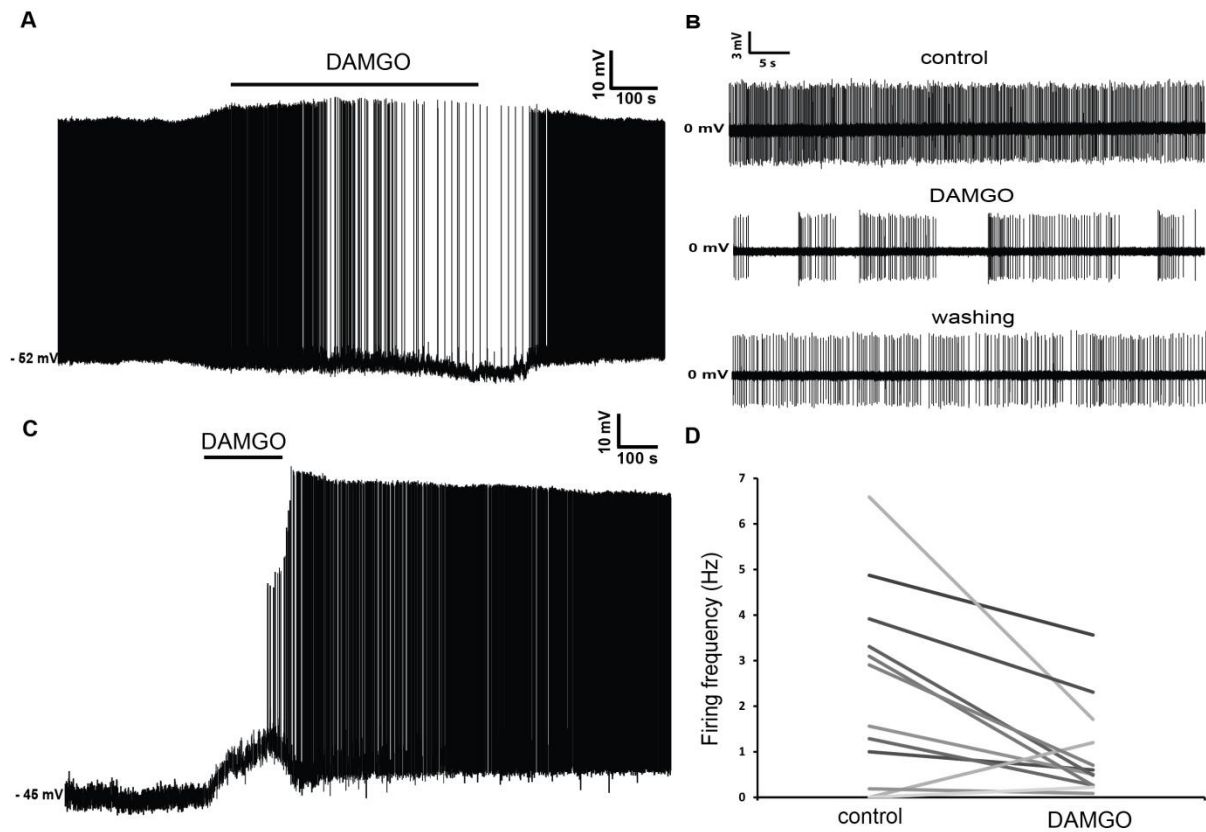


Figure 5.3: Dual effects of DAMGO on the LTSI activity in control solution

A. DAMGO (1 μ M) reversibly blocked LTSI spontaneous activity and caused hyperpolarization in membrane potential (whole-cell recording). B. A cell-attached recording from an LTSI showing reversible depression of DAMGO on spontaneous activity which was reversed by washout. C. In 1/12 DAMGO led to significant ($p < 0.05$) reversible depolarization. D. Effect of DAMGO on firing frequency in individual LTSIs ($n = 12$).

5.3.2.1.2 Effects of μ receptor agonist on LTSI activity in TTX :

In the next group of experiments, the effects of DAMGO on LTSIs in presence of TTX were examined. In 1/7 whole cell recordings, it was observed that DAMGO led to a hyperpolarization of LTSIs membrane potential (Figure 5.4A). On the other hand, in 6/7 experiments, DAMGO application was associated with a depolarization in LTSIs membrane potential (Figure 5.4B). The effects of DAMGO were partially reversed by wash out ($n = 4$) or application of CTOP ($n = 2$). The average change of membrane potential during depolarization was 3.3 ± 1.3 . On the other hand, the average change of membrane potential after recovery was -1.2 ± 0.9 during washout and -1 ± 0.5 with CTOP.

To confirm these results perforated patch technique was used to minimize the intracellular dialysis of the cells. In 4/8 perforated patch recordings, DAMGO caused significant ($p < 0.05$) hyperpolarization of the LTSIs membrane potential. The average change of membrane potential with DAMGO was -3.4 ± 0.6 . After recovery the average of change in membrane potential was 1.6 ± 0.8 with washout ($n = 2$) and 1.8 ± 0.4 with CTOP ($n = 2$). On the other hand, in 4/8 DAMGO led to significant ($p < 0.05$) depolarization of the LTSI membrane potential. The average change of membrane potential was 3.9 ± 3.6 with DAMGO. After recovery the average of change of membrane potential was 2.5 ± 1.9 with washout ($n = 2$) and 2.1 ± 1.3 with CTOP ($n = 2$) (Figure 5.4).

These results confirm the presence of dual effect of DAMGO on LTSIs. The dual effect is more obvious in presence of TTX.

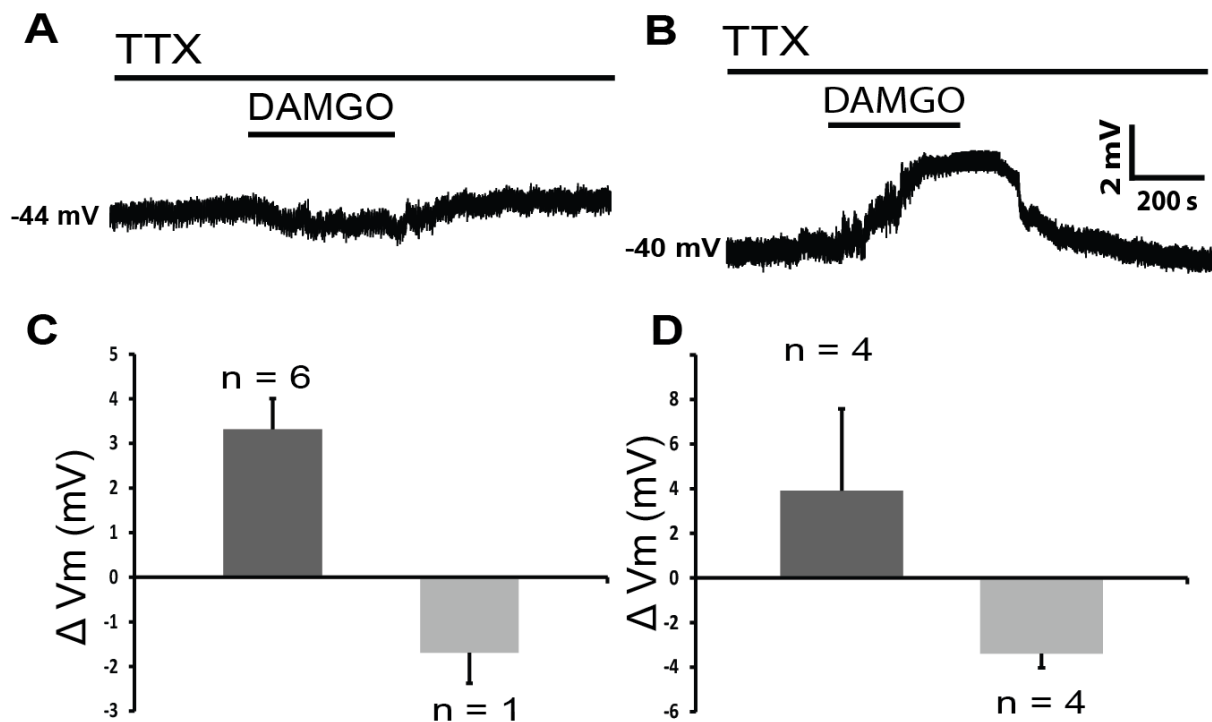


Figure 5.4: Dual effects of DAMGO on the LTSI activity in TTX

A. DAMGO (1 μ M) significantly hyperpolarized LTSIs membrane potential in a LTSI. B. In another LTSI DAMGO caused significant depolarization. C. The average change of the membrane potential in both the depolarization and hyperpolarization states of LTSIs with DAMGO (whole cell recordings). D. The average change of the membrane potential in both the depolarization and hyperpolarization states of LTSIs with DAMGO (perforated patch whole cell recordings).

5.3.2.1.3 Effects of μ receptor agonist on LTSI activity in presence of cholinergic receptor blockers:

In chapter 3, it was reported that LTSIs are under the control of a cholinergic tone and GABAergic tone. Moreover, Ponterio and his colleagues reported that cholinergic interneurons are strongly inhibited by μ receptor agonists (Ponterio et al., 2013). Due to these reasons, the cholinergic interneurons and GABAergic transmission were considered as potential candidates to mediate part of the indirect effects of DAMGO on LTSIs. Understanding the mechanisms involved in these indirect effects were expected to help us in explaining the dual effects of DAMGO on LTSIs.

Accordingly, in one group of experiments, DAMGO was applied in the presence of 1 μ M nicotine and 10 μ M atropine in control solution. In 5/6 whole cell recordings, DAMGO (1 μ M) led to significant ($p<0.05$) decrease in the firing activity of the LTSIs. This effect was accompanied by hyperpolarization of the membrane potential and was reversed by applying μ -receptor selective antagonist (CTOP; 1 μ M) (Figure 5.5A). On the other hand, in 1/ 6 DAMGO application led to significant ($p<0.05$) depolarization of the LTSIs (Figure 5.5B).

For further confirmation of these results, cell- attached recordings and perforated patch technique were used. In perforated patch recordings, 4/6 experiments showed significant ($p<0.05$) hyperpolarization of the LTSIs with significant decrease in their spontaneous firing, while in 2/6 DAMGO led to significant ($p<0.05$) depolarization of the LTSIs. In 1/2 cell attached recordings DAMGO led to significant ($p<0.05$) decrease of the spontaneous firing of LTSIs, while, in 1/2 there was no significant change in the firing activity of LTSIs (Figure 5.5C).

It was concluded that blocking of cholinergic receptors did not stop the dual effect of DAMGO on LTSIs in control solution.

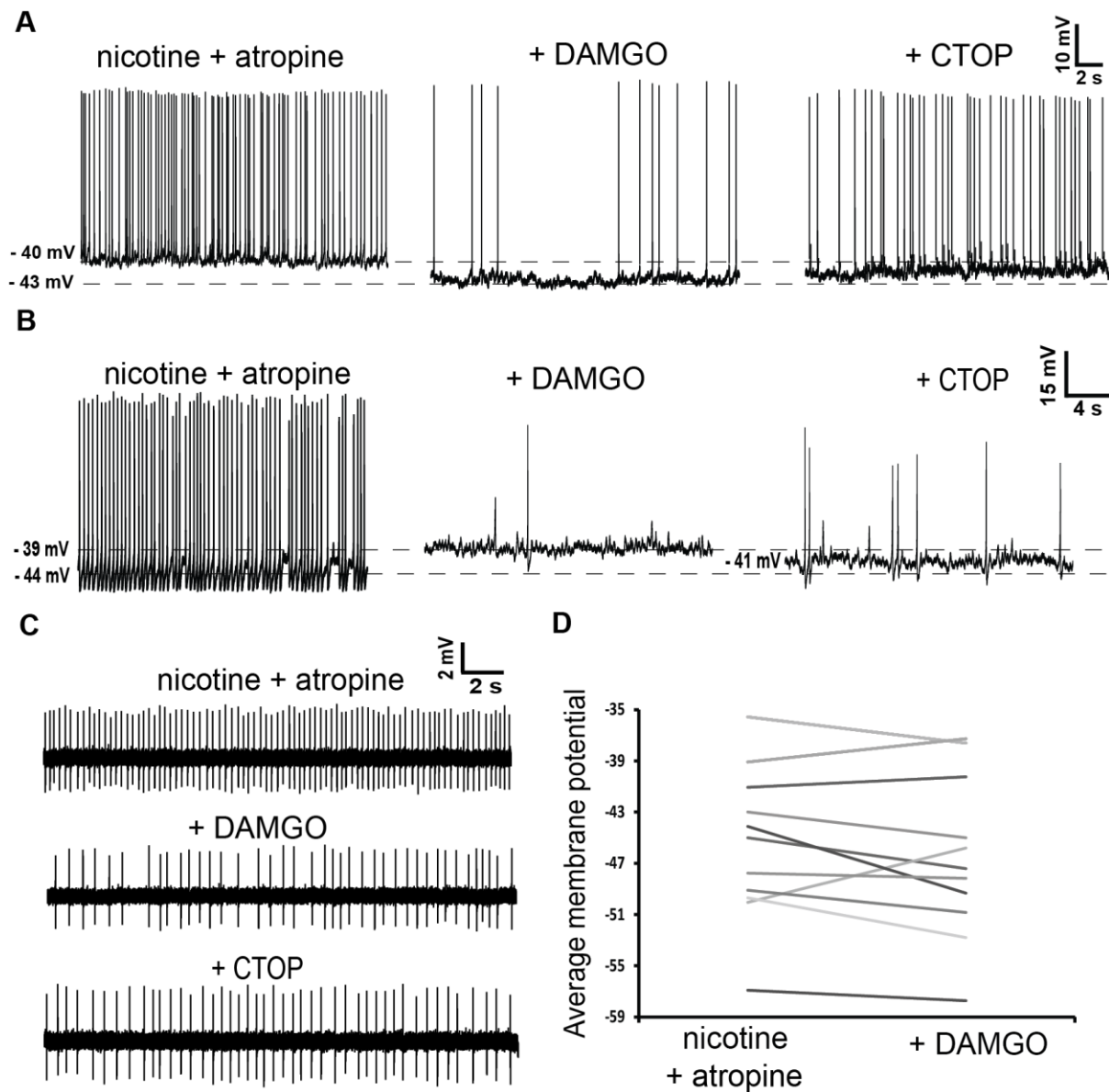


Figure 5.5: Dual effects of DAMGO on the LTSl activity in presence of cholinergic receptors blockers

A. DAMGO (1 μ M) significantly hyperpolarized a LTSl membrane potential ($n = 9$) B. A whole cell recording, DAMGO caused significant depolarization of LTSIs ($n = 3$) C. A cell attached recording shows that DAMGO significantly decreased the spontaneous firing activity of LTSIs (1/2 cells). D. The average change of the membrane potential of LTSIs in presence of DAMGO and cholinergic blockers s ($n = 11$).

5.3.2.1.4 Effects of μ receptor agonist (DAMGO) on GABAergic transmission on LTSIs

As we know that tonic GABA exerts a strong influence on LTSIs. Therefore, the effects of DAMGO on the GABAergic transmission on LTSIs were studied. In the first group of experiments, DAMGO effects on the evoked GABAergic IPSPs in the presence of glutamate blockers were investigated. In 2/4 experiments, DAMGO led to significant ($p < 0.05$) decrease in the evoked GABAergic IPSPs (Figure 5.6 A&B). In the other 2 experiments, DAMGO did not cause any significant change in the IPSPs.

However, we reported in chapter three that GABAergic transmission on LTSIs is modified by the acetylcholine. According to our results, atropine increases the GABAergic transmission while mecamlamine decreases the GABAergic transmission. Thus, in another group of experiments, DAMGO effects on the GABAergic IPSPs in presence of both glutamate receptor blockers and cholinergic receptors blockers were tested. In 2/6 experiments, it was observed that DAMGO led to significant ($p < 0.05$) decrease in the GABAergic IPSPs (Figure 5.6 C&D). In the other 4/6 experiments, DAMGO did not cause any significant change in the GABAergic IPSPs.

This data shows that DAMGO can significantly inhibit the GABAergic transmission on LTSIs in 50% of experiments. However, when cholinergic blockers are added, DAMGO can only inhibit 33% of experiments.

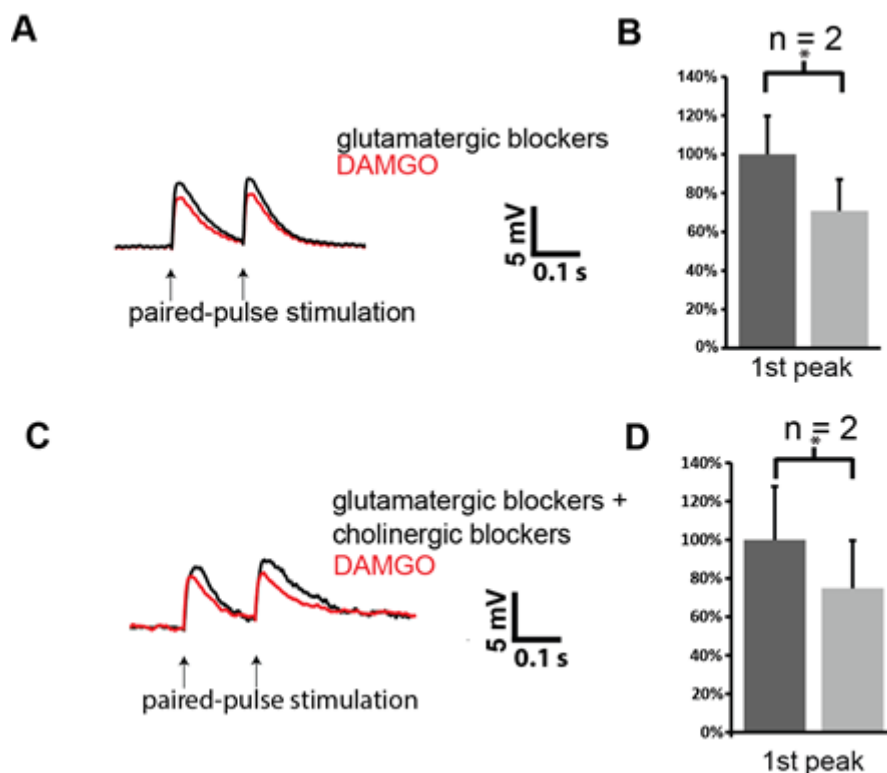


Figure 5.6: DAMGO inhibits the GABAergic transmission on LTSIs.

A. DAMGO (1 μ M) significantly ($p < 0.05$) decreased GABAergic IPSPs in presence of glutamate blockers. B. A diagram shows that DAMGO caused significant decrease in the 1st peak ($n = 2$). C. In presence of cholinergic receptor blockers and glutamate receptor blockers, DAMGO caused significant ($p < 0.05$) decrease in the IPSPs. D. A diagram shows that DAMGO caused significant decrease in the 1st peak ($n = 2$).

5.3.2.1.5 Direct effects of μ receptor agonist on LTSI activity :

From previous data reported in chapter 3, it was concluded that there is tonic GABA and acetylcholine release affecting LTSI activity. Thus, to test the direct effects of DAMGO on LTSIs we had to isolate the LTSIs from the effects of both the cholinergic and GABAergic tones. Thus, in the first group, DAMGO was applied in presence of picrotoxin (100 μ M) and TTX. In 3/5 experiments DAMGO led to significant ($p < 0.05$) hyperpolarization of LTSIs (Figure 5.7A). On the other hand, in 2/5 experiments DAMGO caused significant ($p < 0.05$) depolarization of the LTSIs (Figure 5.7A).

In the second group of experiments, DAMGO was applied in presence of TTX, GABA_A receptors blocker (picrotoxin, 100 μ M) and cholinergic receptors blockers; mecamylamine (10 μ M) and atropine (20 μ M). It was found that in 6/8 experiments DAMGO led to significant ($p<0.05$) hyperpolarization of the LTSIs while in 2/8 there were no significant changes in the membrane potential (Figure 5.7C&D).

We conclude that the direct effect of DAMGO on LTSIs after blocking of GABergic and cholinergic tones is inhibitory.

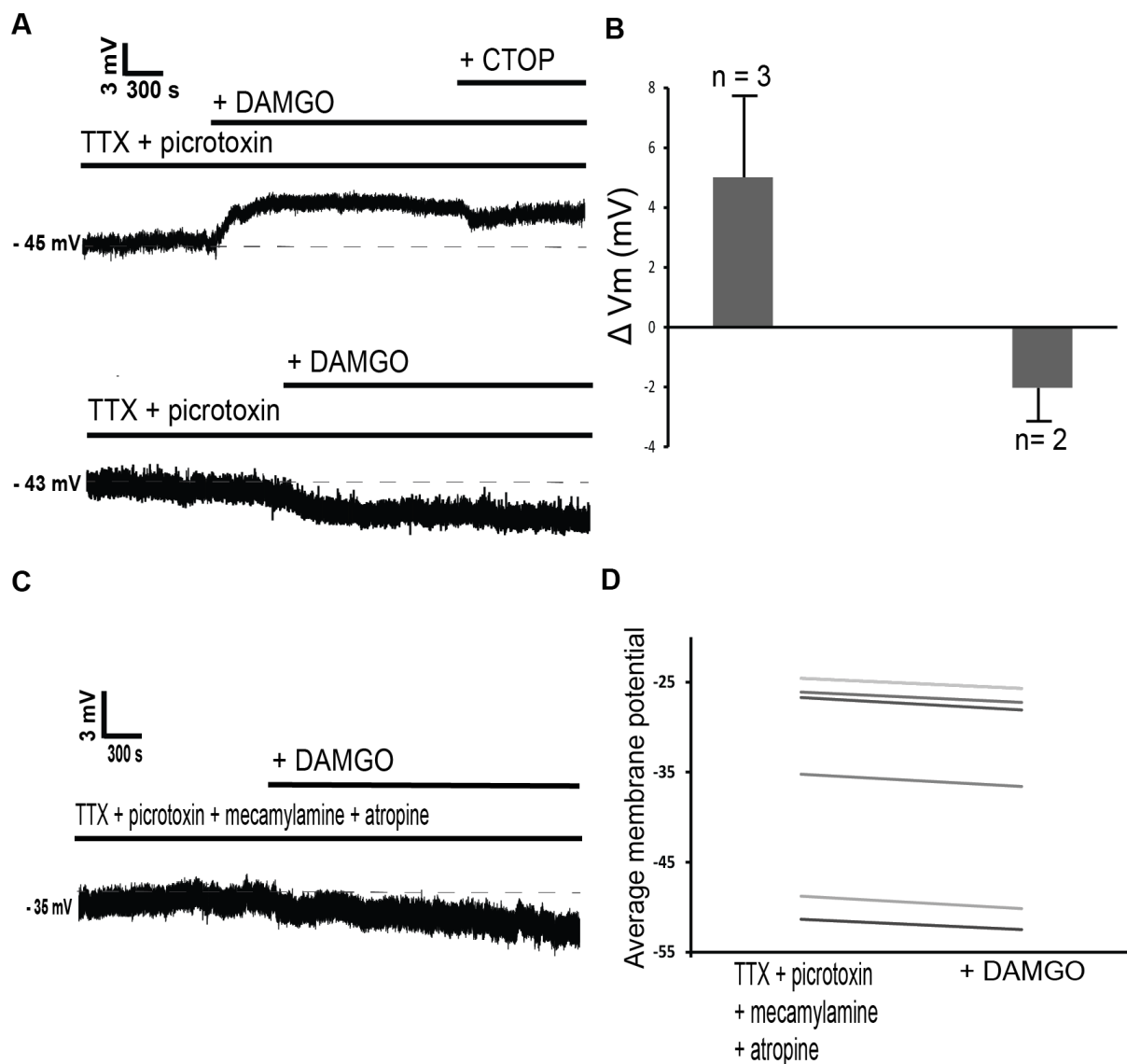


Figure 5.7: Direct effects of DAMGO on LTSIs

A. Dual effects of DAMGO persist in presence of TTX and picrotoxin B. average change of membrane potential with DAMGO in presence of TTX and picrotoxin. C. In presence of TTX, picrotoxin & cholinergic receptor blockers DAMGO inhibited LTSI. D. The changes of the membrane potential in individual cells with DAMGO in presence of TTX, picrotoxin, atropine & mecamylamine) (n= 6).

5.3.2.2 Effects of δ receptor agonists on the LTSI activity:

5.3.2.2.1 Effects of δ receptor agonist on LTSI activity in control & TTX solution :

In the next group of experiments, DPDPE (1 μ M) was used to detect the effect of δ receptor agonists on LTSIs activity. It was observed that DPDPE significantly ($p < 0.05$) inhibited 9/9 LTSIs in whole-cell recordings. DPDPE significantly decreased the spontaneous firing activity of LTSIs and completely stopped the firing of some of these LTSIs (n = 7). This was accompanied by hyperpolarization of the membrane potential. The time between start of application of DPDPE and its maximum effect was on average 27.8 ± 11.3 min. Moreover, the inhibitory effect of DPDPE was partially reversed by wash (n = 7) or by applying δ -receptor antagonist either SDM25aN (1 μ M) (n = 2) (Figure 5.8 A, B).

In the presence of TTX, DPDPE led to significant ($p < 0.05$) hyperpolarization in 5/7 whole cell recordings. In the remaining 2/7 cells, no significant change in the membrane potential was observed (Figure 5.8C).

This data shows that DPDPE exerts strong inhibitory effect on LTSIs directly and indirectly.

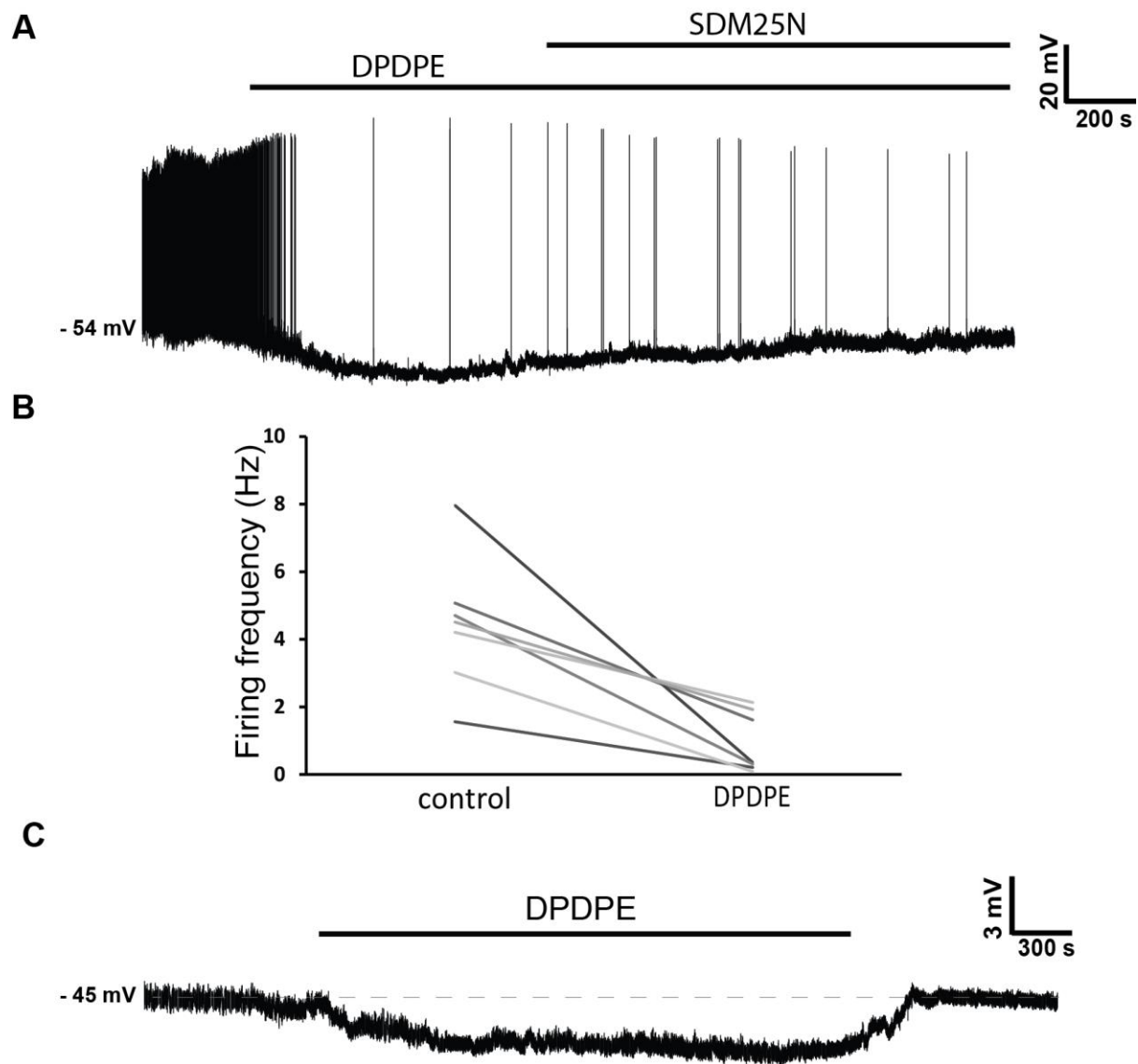


Figure 5.8: DPDPE (1 μ M) inhibits LTSIs activity.

A. DPDPE (1 μ M) significantly ($p < 0.05$) decreased LTSI spontaneous activity and caused hyperpolarization of the membrane potential (whole-cell recording). The effect on membrane potential was partially reversed by applying SDM25N (1 μ M). B. Effect of DPDPE on firing frequency in individual LTSIs ($n = 7$). C. In presence of TTX, DPDPE significantly ($p < 0.05$) hyperpolarized another LTSI ($n = 5$)

5.3.2.2.2 Effects of δ receptor agonist on LTSI activity in presence on cholinergic receptor blockers :

After testing the effects of DPDPE on LTSIs in control solution, we studied the effects of DPDPE on LTSIs in the presence of cholinergic receptor blockers (nicotine & atropine). In 3/4 whole-cell recordings, application of DPDPE (1 μ M) in the presence of nicotine (1 μ M) and atropine (10 μ M) led to significant ($p < 0.05$) inhibition of LTSIs (Figure 5.9A). This inhibition was reversed by applying SDM25N (1 μ M) ($n = 3$). The inhibition was also observed during a cell-attached recording ($n = 2$) (Figure 5.9 B).

These results suggest that the inhibitory effects of DPDPE on LTSIs are not mediated through ACh.

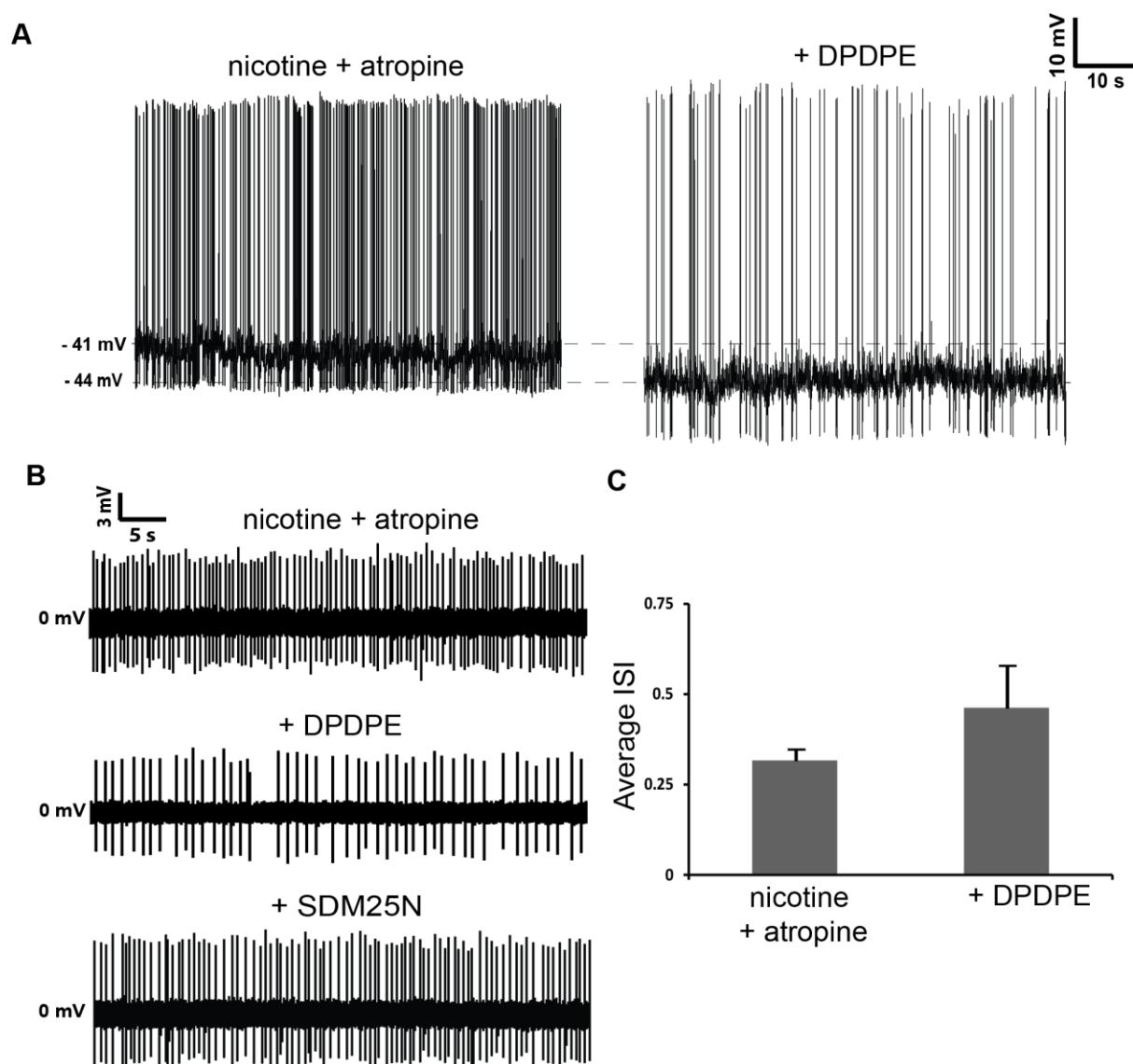


Figure 5.9: DPDPE (1 μ M) inhibits LTSIs in presence of cholinergic receptors blockers.

A. DPDPE (1 μ M) significantly ($p < 0.05$) decreased LTSI spontaneous activity and caused hyperpolarization in membrane potential. The effect on membrane potential was partially reversed by applying SDM25aN (1 μ M) ($n = 3$). B. Effects of DPDPE on spontaneous firing in a cell attached configuration. C. The average change of ISI with DPDPE in presence of cholinergic receptors blockers.

5.3.2.2.3 Effects of δ receptor agonist (DPDPE) on GABAergic transmission on LTSIs

Then, the effects of DPDPE on evoked GABAergic IPSPs were studied. It was observed that in 4/5 experiments, DPDPE led to significant ($p < 0.05$) inhibition of the evoked GABAergic IPSPs. Using Mann–Whitney U test, no significant change in the PPR was observed (Figure 5.10).

It was concluded that DPDPE significantly decrease the GABAergic transmission on LTSIs in 80% of experiments.

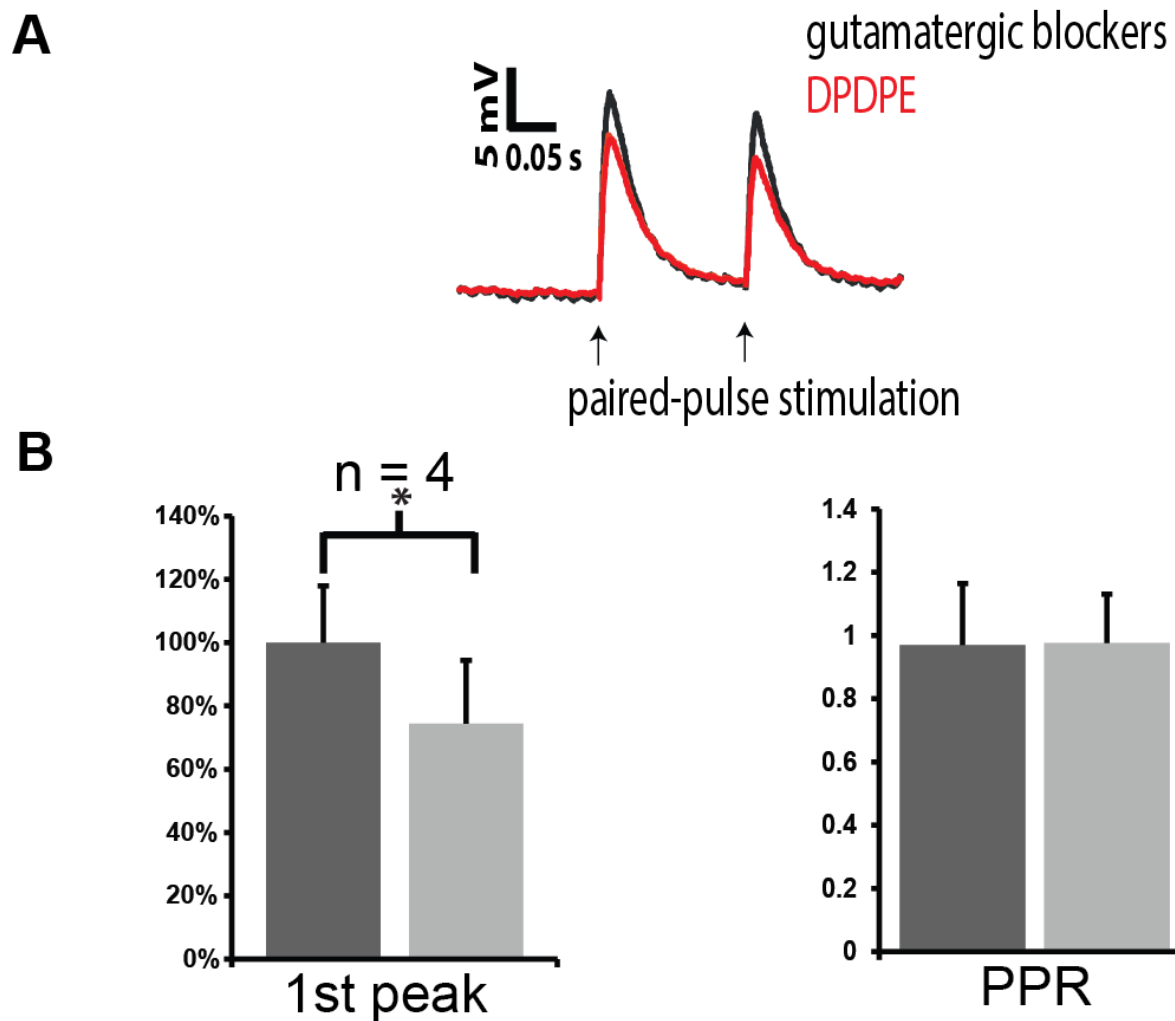


Figure 5.10: DPDPE (1 μ M) inhibits the evoked GABAergic IPSPs on LTSIs.

A. DPDPE (1 μ M) significantly ($p < 0.05$) decreased the evoked GABAergic IPSPs in presence of glutamate blockers in a LTSI. B. A diagram shows the average change in the 1st peak of GABAergic IPSPs with DPDPE (left). Another diagram shows that there is no significant change in the PPR before and after DPDPE application (Mann-Whitney U test) (left).

5.3.2.3 Effects of κ receptor agonists on LTSI activity :

5.3.2.3.1 Effects of κ receptor agonist on LTSI activity in control & TTX solution :

As a κ - receptor agonist, we used U50488H (10 μ M). In 11/12 of whole-cell recordings, U50488H significantly decreased the spontaneous firing activity of LTSIs. The time between start of U50488H application till reaching its maximum effect was on average 18.9 ± 6.1 min. The inhibitory effects of U50488H were partially reversed upon washout ($n = 6$) or by naloxone (20 μ M) ($n = 5$). The average of firing frequency in control was 4 ± 2.7 . With U50488H, the average of firing frequency was 2.5 ± 1.6 . After recovery, the average of firing frequency was 1.5 ± 0.8 with washout and 1.1 ± 0.7 with naloxone. U50488H was able to completely stop the firing activity of some of these LTSIs ($n = 6$). In one experiment the changes in LTSI after application of U50488H were insignificant (Figure 5.11 A).

To confirm these results, we carried out cell-attached experiments. It was found that U50488H caused significant reversible decrease in the firing frequency in 5/6 LTSIs ($p < 0.05$). In the remaining experiment the changes in LTSI activity after application Of U50488H were insignificant (Figure 5.11B).

In the presence of TTX, U50488H caused significant ($p < 0.05$) hyperpolarization in 6/7 whole cell recordings (Figure 5.11D). The effects were partially reversed by washout ($n = 3$) or by naloxone (20 μ M) ($n = 2$). The average change in membrane potential was -4.1 ± 1.3 with U50488H. After recovery the average change in membrane potential was 1.2 ± 0.9 with washout and 1.5 ± 0.4 with naloxone.

It was concluded that U50488H exerts a strong inhibitory effect on LTSIs both directly and indirectly.

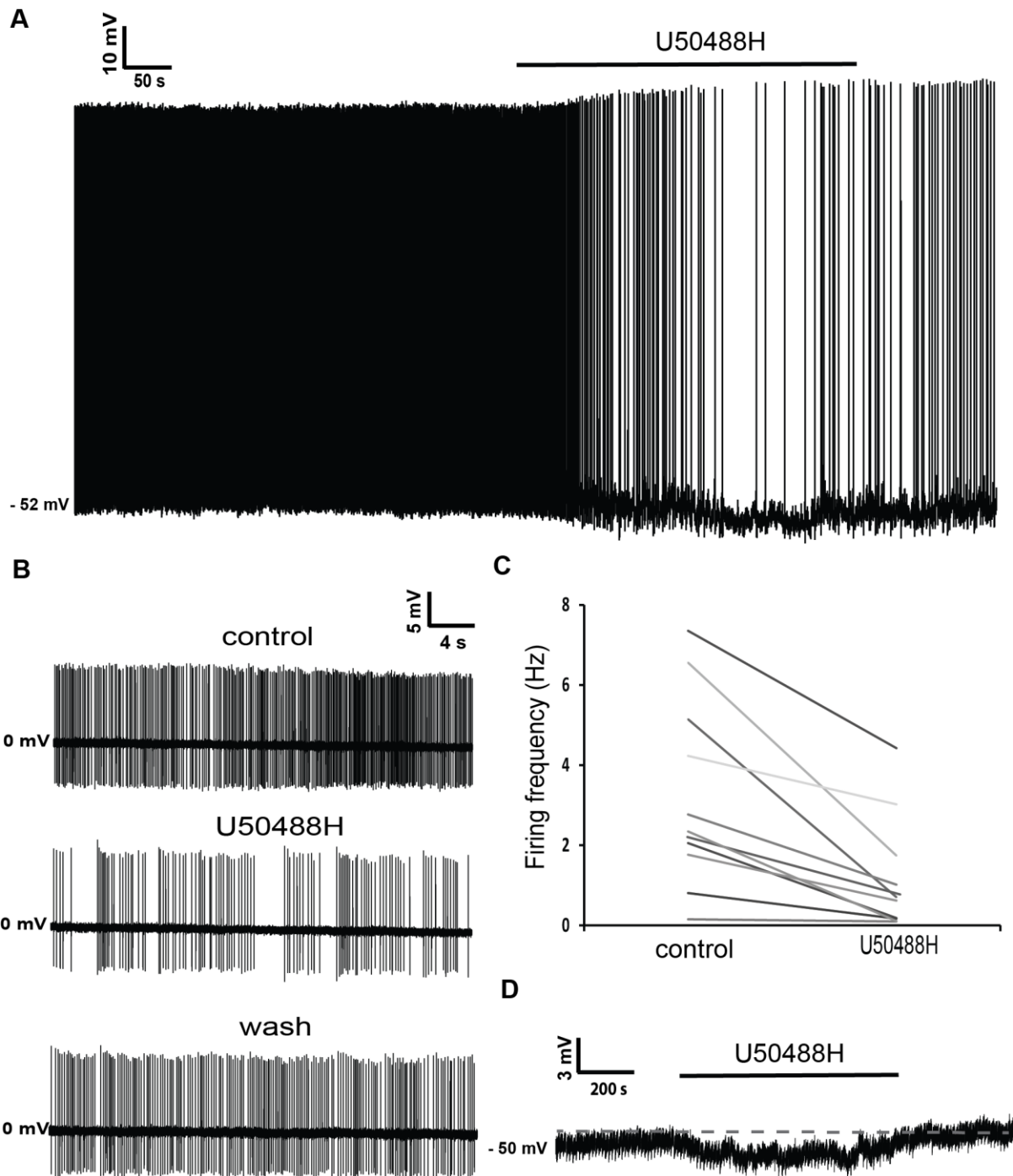


Figure 5.11: U500488H (10 μ M) inhibits LTSIs.

A. U500488H (10 μ M) reversibly blocked a LTSI spontaneous activity and caused hyperpolarization in membrane potential. The effect was partially reversed by washout. B. A cell-attached recording from a LTSI shows that U500488H application caused reversible depression of spontaneous activity that was partially reversed with wash. C. Effects of U500488H on firing frequency in individual LTSIs ($n = 7$). D. U500488H (10 μ M) significantly ($p < 0.05$) inhibited another LTSI in presence of TTX.

5.3.2.3.2 Effects of κ receptor agonist on LTSI activity in presence on cholinergic receptor blockers :

Then, we tested the effects of U50488H on LTSIs in control solution in presence of cholinergic receptors blockers (nicotine & atropine). It was found that, in 4/5 whole-cell recordings, U50488H was still significantly ($p < 0.05$) decreasing the spontaneous firing of LTSIs after blocking of nicotinic and muscarinic receptors (Figure 5.12A). The effect was partially reversed by GNTI dihydrochloride (1 μ M) ($n = 3$). A similar inhibitory response was seen in 2/3 cell-attached recordings ($p < 0.05$) (Figure 5.12B).

This data shows that the inhibitory effects of U50488H on LTSIs are not mediated through a decrease in ACh.

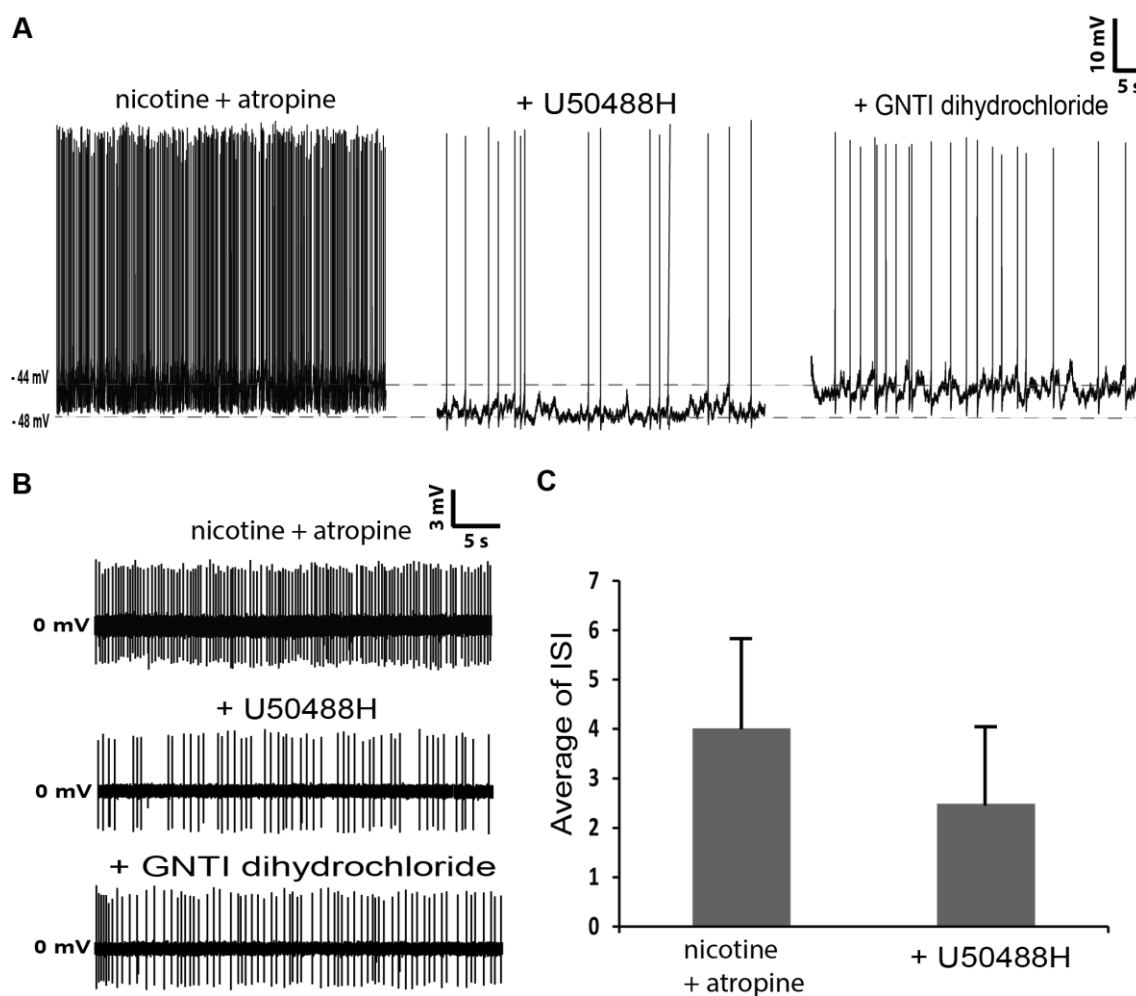


Figure 5.12: U500488H inhibits LTSIs in presence of cholinergic receptor blockers.

A. U50488H (10 μ M) significantly ($p < 0.05$) inhibited a LTSI in presence of cholinergic receptors blockers. B. A cell-attached recording shows inhibition of another LTSI with U5048H in the presence of cholinergic receptor blockers. C. Average change in the ISI in presence of cholinergic blockers only (left) and in presence cholinergic blockers + U50488H (right).

5.3.2.3.3 Effects of κ receptor agonist on GABAergic transmission on LTSIs:

Then the effects of U50488H (10 μ M) on the GABAergic transmission on LTSIs were tested. In 4/4 experiments, U50488H did not cause any significant change in the evoked GABAergic IPSPs (Figure 5.13).

This data suggests that U50488H does not modulate the GABAergic transmission on LTSIs.

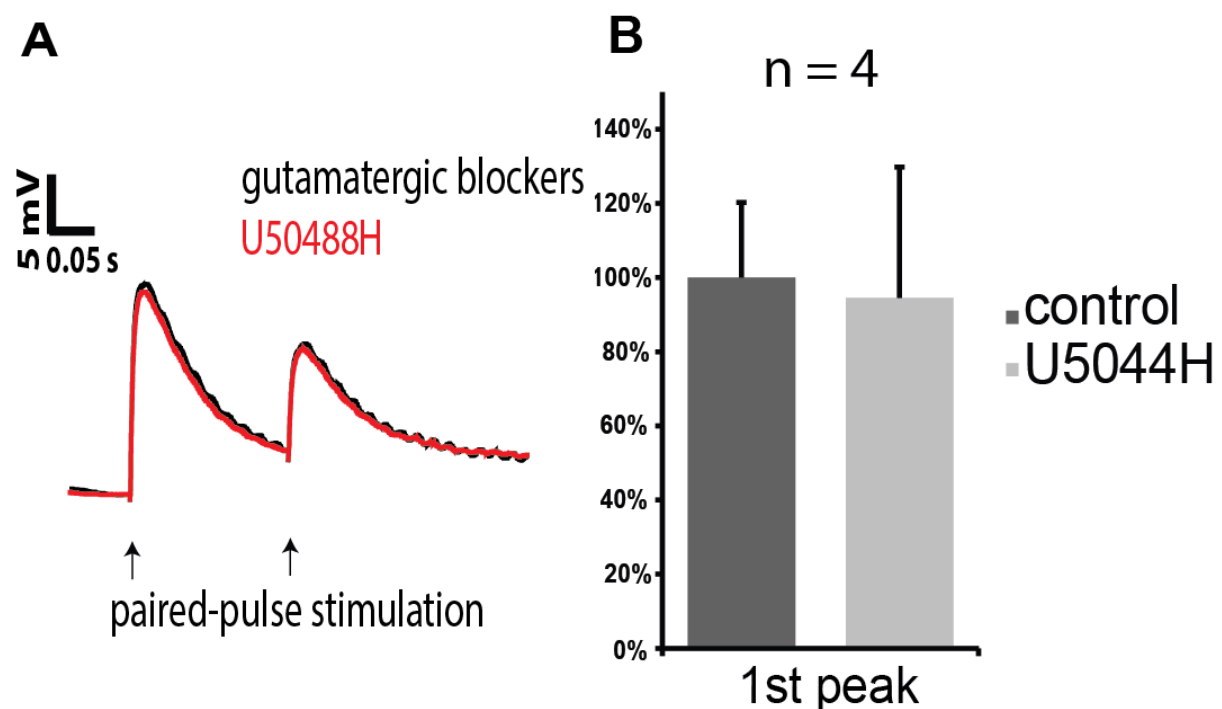


Figure 5.13: U50488H has no effect on the evoked GABAergic IPSPs on LTSIs.

A. U50488H (10 μ M) did not cause any significant change in the evoked GABAergic IPSPs in presence of glutamate blockers. B. The average change in the 1st evoked GABAergic IPSPs with U50488H.

5.4 Discussion:

This study aimed at investigating the effects of activation of different opiate receptors agonists (μ , δ and κ) on cholinergic interneurons and LTSI activity.

In the first part, we studied the effects of opiate receptor agonists on cholinergic interneurons. Cholinergic interneurons represent a potential candidate to mediate the effects of opioids on LTSIs. In the previous chapter, we reported the presence of a cholinergic tone that exerts a powerful modulatory effect on LTSIs through muscarinic and nicotinic receptors. Furthermore, ACh can affect the LTSIs through modulation of the GABAergic transmission on LTSIs.

Moreover, many studies reported relevant results that include : (1) it was found that μ and δ receptors activation on cholinergic interneurons in the NAcc, leads to inhibition of ACh release (Dourmap et al., 1997; Lapchak et al., 1989), (2) cholinergic interneurons act as mediators for the inhibitory effect of opioids on the dopaminergic terminals in the nucleus accumbens (Britt & McGehee, 2008), (3) it was reported that cholinergic interneurons present in the limbic/prefrontal territory of the dorsal striatum express the mRNA and protein of μ opioid receptors. The functional expression of μ receptors follows a diurnal variation (Jabourian et al., 2005), (4) many evidences support that nicotinic and opioid systems interact specially in nicotine addiction. It was proved through studies that acute injections of nicotine in vivo enhance the release of opioids endogenously (Davenport, Houdi, & Van Loon, 1990; Dhatt et al., 1995). Moreover, chronic nicotine administration increases μ receptors expression in striatum of female rats (Wewers, Dhatt, Snively, & Tejwani, 1999). (5) it was reported that MSN collaterals and cholinergic terminals are closely related that suggest close interactions between the cholinergic and opioidergic systems (A. M. Graybiel, Pickel, Joh, Reis, & Ragsdale, 1981), (6) Furthermore, the release of ACh release is decreased after activation of D2 receptors or μ opioid receptors (DeBoer, Heeringa, & Abercrombie, 1996; M Jabourian et al., 2004), and (7) μ receptor agonist causes a powerful inhibition of cholinergic interneurons (Ponterio et al., 2013).

It was found that both DPDPE (δ receptor agonist) and U50488H (κ receptor agonist) inhibited cholinergic interneurons in control solution. We concluded that the three classes of opiate receptor agonists exert strong inhibitory effects on cholinergic interneurons.

Then we studied the effects of opioid receptor agonists on LTSIs. In the first group of experiments the effects of DAMGO (selective μ -receptor agonist) on the LTSIs were investigated. In control solution, it was found that DAMGO led to significant inhibition of the LTSIs in 73% of experiments. However, in about 6% of the experiments, DAMGO led to depolarization of the LTSIs. In the presence of TTX, DAMGO caused a depolarization in two thirds of tested LTSIs. On the other hand, one third of LTSIs were hyperpolarized with DAMGO.

We hypothesized that this dual effect of DAMGO on LTSIs could be due to different mechanisms through which DAMGO affects LTSI activity directly and indirectly. The indirect effects of DAMGO could be mediated by cholinergic interneurons and GABA transmission on LTSIs.

In the presence of cholinergic receptor blockers only, it was observed that DAMGO has dual effects on LTSIs. We also found that DAMGO significantly decreased the GABAergic transmission on LTSIs in 50 % of experiments. When the cholinergic receptors were blocked, DAMGO had a significant inhibitory effect on the GABAergic transmission in only one third of experiments.

In the presence of TTX, we examined the effects of DAMGO after blocking of the GABAergic tone. It was found that DAMGO was still able to cause both depolarization (50% of experiments) and hyperpolarization (50% of experiments) of the LTSI. However, when cholinergic blockers were added to GABA_A blocker and TTX, DAMGO caused inhibition in 100% of the experiments.

These results suggest that DAMGO exert direct inhibitory effects on LTSIs. However, it also inhibits the cholinergic interneurons and GABA transmission on LTSI. According to the final balance between these direct and indirect effects of DAMGO, it could lead to either an inhibition or activation (through release of inhibition) of the LTSIs.

In the second group of experiments, we tested the effects of DPDPE (selective δ -receptor agonist) on LTSIs. DPDPE caused inhibition of LTSIs in both control solution and in TTX in 100% of experiments. Furthermore, DPDPE inhibited evoked GABAergic IPSPs on LTSIs in 80% of tested LTSIs.

In the third group of experiments, we tested the effects of U50488H (selective κ -receptor agonist) on LTSIs. U50488H inhibited LTSIs in both control and in presence of TTX in 100% of experiments. However, U50488H did not affect the GABAergic transmission on LTSIs. We conclude that δ and κ agonist main effect on LTSIs is inhibition. This inhibitory effect is mediated both directly and indirectly mainly through inhibition of the cholinergic transmission. Moreover, it seems that δ agonists have a weak inhibitory effect on GABAergic transmission on LTSIs. On the other hand, κ agonists have no significant effect on GABAergic transmission.

Moreover, it was observed that the inhibitory effect of opioid receptor agonists developed gradually and lasted for a long time. Full recovery of LTSIs did not happen in any experiment either by washout or applying the selective antagonists. This long action time may be due to triggering of slow cellular responses in the LTSIs by opioids. It was previously reported that μ , δ and κ opioid receptor agonists produce long term depression (LTD) of excitatory inputs in the dorsal striatum (Atwood, Kupferschmidt, & Lovinger, 2014). This LTD can give a possible explanation for long-term effect of drugs of abuse on neuronal activity.

These results improve our understanding of the modulatory role of opiates on the striatal microcircuit. The implications of the experimental findings in this chapter on the basal ganglia function and pathology will be fully explored in the final discussion chapter of this thesis.

Chapter Six: General discussion

In this chapter, the main findings of this study will be summarized. Also, I will throw light on the expected implications of these results on our understanding of the basal ganglia function. Moreover, the technical limitations will be discussed. Finally, suggestions for the future work will be mentioned.

6.1 The mutual interaction between LTSIs and cholinergic interneurons

In the chapter 3 and 4 of this thesis, we examined the mutual interaction between cholinergic interneurons and LTSIs. Understanding this interaction will definitely deepen our understanding about the functionality of striatal network.

LTSIs and cholinergic interneurons are the only known TANs inside the striatum. In-vivo studies reported an important role played by TANs in reward processing through TAN pause response (Aosaki, 1994). However, In-vivo studies were always interpreted on the basis that cholinergic interneurons are the TANs in the striatum.

Our results provide an overview of the relationship between cholinergic interneurons and LTSIs in-vitro. This data prove that LTSIs are under a cholinergic control that modulates their activity directly through muscarinic and nicotinic receptor on LTSIs and indirectly through modification of the GABAergic transmission on LTSIs. Moreover, our results shows that there is a cholinergic and GABAergic tones that keep the LTSIs under control even with absence of action- potential released transmitters. On the other hand, LTSIs exert a stimulatory effect on cholinergic interneurons through NO (Figure 6.1).

Our results provide an overview of the interaction between LTSIs and cholinergic interneurons in-vitro. This data provide strong evidence that LTSIs are under a cholinergic control that modulates their activity directly through muscarinic and nicotinic receptor on LTSIs and indirectly through modification of the GABAergic transmission on LTSIs. Moreover, our results shows that there is a cholinergic and GABAergic tones that keep the LTSIs under control even with absence of action- potential released transmitters. On the other hand, LTSIs exert a stimulatory effect on cholinergic interneurons through NO (Figure 6.1).

The initial activation of cholinergic interneurons tends to activate LTSIs. LTSIs can activate the cholinergic interneurons through NO. However, LTSIs secrete other neurotransmitters that are mainly inhibitory such as GABA and somatostatin. Moreover, activation of this part of the striatal network is expected to affect the activity of other players in the striatal network. Thus, the initial activation of cholinergic interneurons by LTSIs cannot establish a positive forward feedback loop between the two interneurons. It is expected that the initial activation of cholinergic interneurons will eventually result in inhibition of the cholinergic interneurons at some point. This inhibitor effect could be mediated by LTSIs or other players in the network.

These finding can be linked to the TANs pause response. TANs respond to stimuli associated with behavioural value (reward). It is believed that TANS play an important role in the selection of appropriate response to any environmental stimuli (Paul Apicella, 2007). The response of TANs consists of three phases: initial activation, inhibition and rebound activation (Toshihiko Aosaki et al., 1994). It was noted that during TANs activation, the dopamine levels decrease dramatically (Toshihiko Aosaki et al., 2010). From our results we showed how LTSIs and cholinergic interneurons activate each other. This means that once one or both of these two cells are activate by salient stimulus, the positive feedback loop between the two cells will be activated leading to potentiating their response. By other words, salient stimuli will trigger the release of more acetylcholine and NO in the striatum. This increase will lead to decreased dopamine release.

NO is known a role in synaptic plasticity (Susswein et al., 2004). Moreover, TAN pause response act as a time window for synaptic plasticity in the striatum (Miura et al., 2007). LTSIs are expected to be involved in the response to salient stimuli either directly or indirectly through activated cholinergic interneurons. We can assume that LTSIs activation during response in reward will not only share in the immediate response to the environmental stimuli but also it will share in the learning and memory aspects through NO on synaptic plasticity.

Moreover, by being closely working together, LTSIs and cholinergic interneurons will act as fine tuners for the striatal output through the many transmitters released by the two cells that include: NO, acetylcholine, GABA, NPY and somatostatin.

Although this study focused on the direct mutual interaction between cholinergic interneurons and LTSIs, the role of other players of striatal network cannot be ignored. In

each state when the activity of the cholinergic or the LTSIs interneurons will be modified this will be reflected on the activity of the other neurons in the striatal microcircuitry.

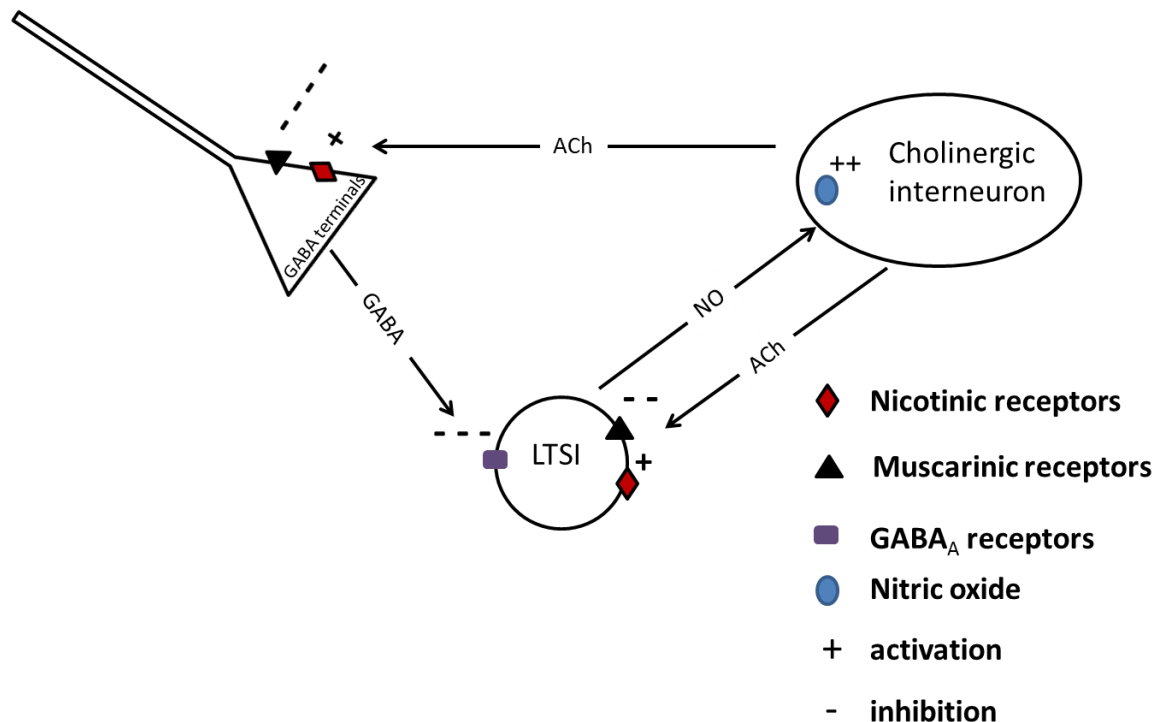


Figure 6.1: Schematic diagram explains the mutual control between cholinergic interneurons and LTSIs

The diagram summarizes the main findings in this study about the mutual interaction between cholinergic interneurons and LTSIs. The cholinergic interneurons modulate LTSIs through ACh both directly and indirectly. Directly ACh can activate LTSIs through nicotinic receptors or inhibit LTSIs through muscarinic receptors. Indirectly, ACh can increase GABA release from GABA terminals through nicotinic action leading to inhibition of LTSIs. On the other hand, ACh can inhibit GABA release through muscarinic action (dominant effect). On the other hand, LTSIs release NO that activates cholinergic interneurons.

6.2 The effects of opiate peptides on cholinergic interneuron and LTSI activity.

In the second part of this study, we investigated the effects of different opioid peptides on the LTSI activity.

Our results show that different types of opiate peptides exert a direct inhibitory effect on the LTSIs. Moreover, μ and δ receptor agonists exert inhibitory effects on both the cholinergic interneurons and GABAergic transmission on LTSIs. While κ receptor agonist inhibited the cholinergic interneurons but it did not significantly affect the GABAergic transmission (Figure 6.2).

Although being mainly inhibitory in action, we reported that μ receptor agonist can lead to an activation of the LTSIs through disinhibiting the inhibitory inputs from GABAergic and cholinergic interneurons. This stimulatory effect of μ receptor agonist is greater in TTX than in control solution. In TTX, LTSIs are isolated from the synaptic influence of the surrounding neurons. However, even with blocking the action potential – dependant release of neurotransmitters, we reported that LTSIs are still under the control of a tonic release of ACh and GABA. In this condition, it seems that DAMGO inhibitory effect on the GABAergic and cholinergic tone is stronger than the direct inhibitory effect on LTSIs. This can explain the higher possibility of LTSIs depolarization with DAMGO.

Generally, the opioidergic control of LTSIs (either through activation or inhibition) is expected to modulate the levels of the neurotransmitters released by these neurons. LTSIs are known to be the only source for NO in striatum. NO inhibits glutamate transporters leading to an increase in extracellular glutamate concentrations (Taskiran et al., 2003). Moreover, it increases glutamate release in the dorsal striatum and nucleus accumbens (Lonart & Johnson, 1994; S. Pogun, V. Dawson, & M. J. Kuhar, 1994; Taskiran et al., 2003). Also, NO increase the firing rate of cholinergic interneurons that will increase acetylcholine levels in the striatum.

Another transmitter released by LTSIs is GABA. GABA decrease expected to affect activity of other striatal neurons especially the MSNs. GABA released by LTSIs mediates feed-forward inhibition of MSNs (Koos & Tepper, 1999). So, the change in the GABA levels is expected to affect MSNs activity.

Moreover, somatostatin is released by LTSIs. Somatostatin modulates the firing pattern of MSNs (Galarraga et al., 2007; Vilchis et al., 2002), and the effectiveness of lateral synaptic connections between them (Lopez-Huerta et al., 2008). This means that when opioids inhibit the LTSIs, the excitability of MSNs will increase due to decreased levels of somatostatin.

So, from clinical point of view, drugs of abuse could inhibit LTSIs leading to an increase in MSNs activity due to decreased GABA and somatostatin levels. This can explain the motor side effects associated with intake with these drugs.

Finally, opioids can modulate the levels of NPY, which is released by the LTSIs. NPY proved to increase dopamine overflow through activation of NPY- Y_2 receptor subtype (Adewale, Macarthur, & Westfall, 2007).

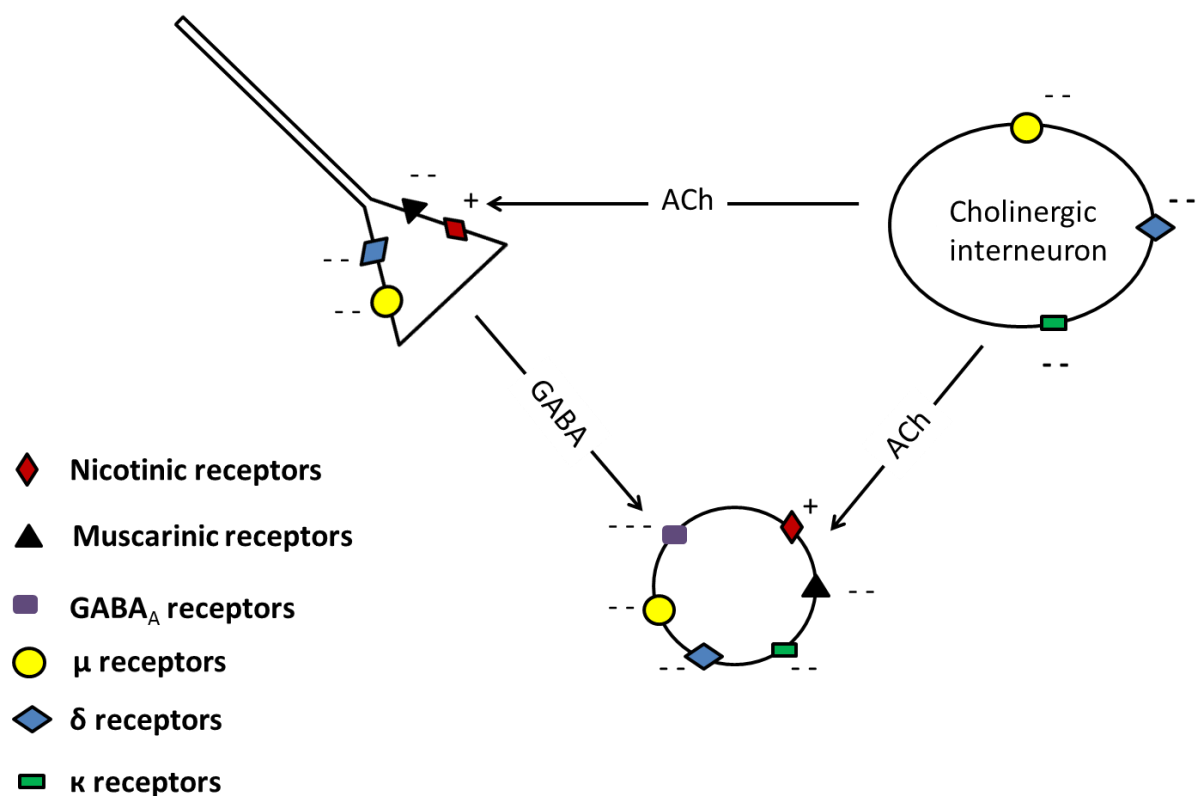


Figure 6.2: Schematic diagram explain the opioidergic control of cholinergic interneurons and LTSIs

The diagram summarizes the modulation of cholinergic interneurons activity and LTSIs activity through different subtypes of opiates. μ -opioid receptor agonists inhibit both cholinergic interneurons and LTSIs directly. Moreover, μ opioid receptor agonists inhibit the GABAergic transmission on LTSIs. The net effect of μ -opioid receptor agonists on LTSIs will depend on the balance between their direct and indirect effects. δ receptor agonists inhibit both cholinergic interneurons and LTSIs. Moreover, δ receptor agonists inhibit the GABAergic transmission on LTSIs. Finally, κ receptor agonists inhibit the cholinergic interneurons and LTSIs. However, we couldn't find any significant effect of κ receptor agonists on GABAergic transmission on LTSIs.

6.3 Technical considerations

The main focus of my research project was to study the interaction between LTSIs and cholinergic interneurons. Also I investigated the effects of different opiate peptides on the activity of cholinergic and LTS interneurons.

To approach this complex research field, I have used several electrophysiological approaches. For instance, I successfully used conventional whole cell patch clamp recordings. I also used the perforated cell patch clamp technique and cell-attached patch clamp recordings. Moreover, I used single patch clamp recordings together with electrical activation of axons to study the presynaptic inhibition and activation.

The main technique utilised in this study was whole cell recordings of neurons. Whole-cell recordings provide electrical access to the cell cytosome, allowing direct recording of the internal electrophysiological state (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981; Sakmann & Neher, 1984). Whole-cell recordings are characterized by providing accurate measurements of cell electrophysiological properties. However, this comes at the expense of disruption of the cell internal milieu by the dialysis of the internal pipette solution into the neuron (Sakmann & Neher, 1984). Therefore, it is essential to closely monitoring the neuron state throughout the experiments, discarding neurons when their electrophysiological properties are altered (e.g. input resistance and spike intensity)

Moreover, perforated whole-cell patch technique was also used in this study. Perforated whole-cell patch is a powerful electrophysiological approach that allows recording the membrane potential of a neuron without interfering with its intracellular milieu. Amphotericin was used as a pore forming agent. Pores in the membrane are usually formed within 15 minutes from the approaching the cell (Rae, Cooper, Gates, & Watsky, 1991). Amphotericin is light sensitive, thus all precautions were taken to avoid light either during the preparation of the drug or during the patching process. It was challenging to carry the whole procedure in darkness taking in consideration all health and safety measures.

Furthermore, we used cell- attached technique. This approach is useful in studying the extracellular activity of the cell without interfering with the cell intracellular milieu. LTSIs and cholinergic interneurons are spontaneously active cells which make cell- attached recording a powerful tool to study the effects of different ligands on their activity.

Generally, the patch-clamp technique is suitable for investigating the molecular mechanisms of receptors. The advantages of the patch-clamp method include: (a) Any pharmacological agent can be easily applied either in the bath or in the pipette, in the desired concentrations, (b) several chemical agents can be tested either together or in sequence; and (c) different concentrations of pharmacological agents can be tested on the same membrane patch, thus enabling the generation of dose–response curves. Moreover, the mechanisms underlying the opening of different ion channels can be easily tested pharmacologically (Blanton, Turco, & Kriegstein, 1989; Grantyn & Kettenmann, 1992; Sakmann & Neher, 1984).

The main restriction of the patch-clamp method is the necessity to use fresh brain slices. This process of the preparation of these slices includes many factors that need to be adjusted. These factors include the preparation of suitable solutions to keep the slices, adjusted PH and osmolarity, good oxygenation of used solutions and suitable temperature for slices. Moreover, with patch-clamp technique cell is exposed to "run-down" due to diffusion of cell constituents into the pipette. This process of run-down is minimized in perforated –patch and cell-attached recordings (Blanton, Turco, & Kriegstein, 1989; Grantyn & Kettenmann, 1992; Sakmann & Neher, 1984).

Although in-vivo studies provide the opportunity to study brain regions in their intact state, however, there are many disadvantages of in vivo preparations. It is relatively difficult to study the mechanisms of drug action in vivo. Moreover, the presence of anaesthetics could affect the normal electrophysiological responses to drugs and transmitters. Finally, it is

sometimes difficult to identify the drug concentrations at the neurons under study accurately (Blanton, Turco, & Kriegstein, 1989; Grantyn & Kettenmann, 1992; Sakmann & Neher, 1984).

From another prospective, part of this research project was carried out using optogenetics. The optogenetic revolution of the last years has provided a promising approach in the field of electrophysiology. Stimulation of neurons through optogenetics has many advantages over traditional electrical stimulation. Optogenetic stimulation allows greater numbers of neurons to be stimulated simultaneously. Also, optogenetics ensure specific stimulation of cells that express light-responsive proteins (Fields, 2007).

In this research project, the effects of activation of LTSIs on the cholinergic interneurons were studied through optogenetics. Two colonies of transgenic mice (SOM-IRES-Cre mice and ChR2 (H134R)-EYFP mice) were crossed together to get a heterozygous transgenic ChR2-EYFP-SOM-IRES-Cre mice colony.

Although optogenetics represent a promising tool for electrophysiology, many improvements are still needed that include: 1.) Transfection methods; the molecular biology construction of rhodopsins is challenging due to the presence of several variety of vectors and promoters that have to be screened. On the other hand, the construction of transgenic animals is a time consuming process that need breeding of several generations before obtaining the desired offsprings. 2.) Improvement of the light sensitivity of ChR2 and NphR is important for experiments on the mammalian brain because of its the low transmittance. 3.) Improvement of light emission diodes (LED's) for better spatial resolution. An alternate approach is to design of arrays of micro light pipes that provide sufficient for light stimulation on brain in the submillimeter range (Zhang et al., 2010).

6.4 Future experimental work

The results of this thesis raise a number of important questions that need to be addressed in future series of experiments.

For example, it will be interesting to study the conductances and intracellular mechanisms underlying the different reported findings. Moreover, it is also recommended to study of the distribution of the different types of cholinergic receptors (muscarinic, nicotinic) and opioid

receptors (μ , δ , and κ) on different striatal neurons. Immunohistochemical studies represent the best technique to achieve this goal.

Furthermore, a lot of information about the striatal microcircuits could be obtained using optogenetics. Breeding of different colonies of mice with different distribution of ChR2 and NphR on different neurons will provide an important approach to study the interaction between different striatal neurons. ChAT-Cre mice strain would allow us to study many interactions between cholinergic interneurons and other striatal neurons specially MSNs. The optogenetic method can provide new opportunities to analyse neural networks.

In-vivo recordings are also potential approach. It was previously reported that during slow-wave activity (SWA), cholinergic interneurons, and some LTSIs were tonically active. Each interneuron type exhibited distinct responses to cortical stimulation in vivo (Sharott et al., 2012). Using optogenetics mice in which specific expression of ChR2 and NphR on the cholinergic and/or LTSIs will give more information about the behaviour of these cells in relation to the each other and in relation to other striatal neurons.

Finally mathematical modelling is a powerful tool that can use different pieces of information together to predict the way the network will behave in different conditions. Results of this thesis add information that can be used during the construction of models of the striatal network. Having in-vivo and in-vitro electrophysiological recording for the TANs with using the optogenetic activation and inhibition will provide important data that will help in building a model that can predict the response of striatal network to different stimuli in normal and pathological conditions. Computational experiments based on such models of cholinergic and nitrgergic transmission could predict important information about: (a) time binding of acetylcholine to different cholinergic receptors; (b) open probability of the different receptors; and (c) single receptor conductance. Moreover, the data provided in chapter 5 on opiate receptors could be used to construct a model for synaptic transmission of opioids during normal processing of reward and in cases of drug abuse.

6.5 Concluding remarks

Although there have been many developments in the field of BG research over the past 20 years. However, a lot of information about the functionality of the different parts of BG is still missing.

This thesis cast the light on different novel interactions affecting striatal network. Also, this thesis highlights the role of many neurotransmitters such as acetylcholine, GABA, NO and opiate peptides in modulation of striatal neurons. Furthermore, this thesis provides clear directions for future experimental work in this field.

Unveiling more details about the processing of information through BG network will provide new insights for the treatment of disease states affecting this part of the brain.

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